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Adaptation of *Candida albicans* to Reactive Sulfur Species

by

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Abstract

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The opportunistic fungal pathogen *Candida albicans* is both a common commensal of the human microbiota and an agent of fatal systemic infections. *C. albicans* inhabits the mouth and skin, as well as gastrointestinal and genitourinary tracts of humans. One attribute that allows *C. albicans* to inhabit such physiologically distinct niches is the ability to resist and adapt to various oxidative stresses. This includes stress caused by reactive sulfur species (RSS), such as sulfite, produced by *C. albicans,* sulfate-reducing commensal microbes, and the host immune system. From a collection of transcription factor deletion mutants, only the mutant lacking the zinc cluster factor gene *ZCF2* was found to be specifically sensitive to sulfite. In my thesis I show that *C. albicans* distinctively adapts to sulfite stress, and that Zcf2, as well as the sulfite exporter, Ssu1, are required for that response.

Gene expression profiling revealed that Zcf2 is required for the induction of genes predicted to remove sulfite from cells, and increase the import of a subset of nitrogen metabolites. Additionally, analysis of mutants in the sulfate assimilation pathway show that sulfite conversion to sulfide accounts for part of sulfite toxicity and that Zcf2-dependent expression of *SSU1* is induced by both sulfite and sulfide. Mutations in the *SSU1* promoter that selectively inhibit induction by sulfite, or the reactive nitrogen species (RNS), nitrite, a

previously reported activator of *SSU1,* led to the identification of distinct cis-acting regions in the *SSU1* promoter. This supports a model in which RNS and RSS-induction of *SSU1* are mediated by parallel pathways. Lastly, I found that endogenous sulfite production leads to an increase in resistance to exogenously added sulfite. Taken together, these data demonstrate that *C. albicans* has a unique response to sulfite that differs from the general oxidative stress response, and that adaptation to internal and external sulfite is largely mediated by one transcription factor and one effector gene.

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Abbreviations

APS	5’-Adenylylsulfate
BSI	Blood stream infection
GSH	Glutathione
kb	Kilobase
ORF	Open reading frame
PAPS	3’-Phospho-5’-adenylylsulfate
PCR	Polymerase chain reaction
RLU	Relative luminescence units
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSS	Reactive sulfur species
SD	Standard deviation
SEM	Standard error of the mean
SOD	Superoxide dismutase
TF	Transcription factor
UTR	Untranslated region
ZCF	Zinc cluster family

Chapter 1: Introduction

Mithridates VI of Pontus, one of Rome's deadliest enemies, and a polyglot speaking more than 20 different languages, is most famously remembered for being immune to poison (MAYOR 2011). Per Appian's *Roman History* he had "accustomed himself to other drugs by continually trying them as a means of protection against poisoners." I began my research by observing how yeast is more resistant to certain toxins after being exposed to sub-lethal amounts of the same toxins, in likeness to how Mithridates became resistant to his poisons. Although I do not understand the mechanisms behind the poison king's tolerance to poison, which took him years to develop, I do have a better understanding of the stress adaptation processes used by the yeast. These processes take minutes to occur, and nonetheless it takes years to study them. This doctoral thesis will describe in intense and fascinating detail how *Candida albicans* adapts to one of the poisons it encounters, sulfite.

1.1 *Candida albicans* as a commensal fungus and opportunistic pathogen.

Although there is a vast number of fungal species on earth, only 600 species are known human pathogens (MORA *et al.* 2011; BROWN *et al.* 2012; MAYER *et al.* 2013). Fungal diseases range from superficial infections, affecting the mucosa and skin, to serious invasive fungal infections that affect deep tissues and organs. While the selection of fungal pathogens known to cause invasive infections is very diverse, most of these infections are caused by *Candida* spp. (PERLROTH *et al.* 2007; PFALLER AND DIEKEMA 2010). *Candida* species account for approximately 9% of all hospital acquired bloodstream infections (BSI), making them the fourth leading cause of nosocomial BSIs (WISPLINGHOFF *et al.* 2004). The crude

mortality rate of *Candida* BSI, or candidemia, is reported around 40%, although rates might be as high as 50-60% (WENZEL AND GENNINGS 2005; MIKULSKA *et al.* 2012). *Candida* infections are particularly serious in immunocompromised people and healthy people with implanted medical devices (KULLBERG AND OUDE LASHOF 2002; KOJIC AND DAROUICHE 2004). Although there are more than 100 *Candida* species described, less than 30 of them cause disease in humans, the most common of which is *Candida albicans* (PFALLER AND DIEKEMA 2010).

Candida albicans is the most prevalent fungal species of the human microbiota, asymptomatically inhabiting the gastrointestinal tract, reproductive tract, oral cavity, and skin of most humans (ACHKAR AND FRIES 2010; GANGULY AND MITCHELL 2011; NOBILE AND JOHNSON 2015). Even though *C. albicans* commonly exists as a commensal, it is the predominant cause of invasive fungal infections and serves as a serious public health challenge due to the high mortality rates and increased costs of hospitalization (ALMIRANTE *et al.* 2005; HORN *et al.* 2009; SARDI *et al.* 2013). Increasing resistance of *C. albicans* to current antifungal therapies, particularly those involving the azole class of antifungals, is also of growing concern. A recent retrospective case study conducted at two cancer centers in Boston reported that 19% of their *Candida* infections involved fluconazole-resistant strains or strains with reduced susceptibility (OXMAN *et al.* 2010). Several azole resistance mechanisms have been described in *C. albicans;* their discoveries are in no small part due to the basic understanding of *C. albicans* virulence in relation to its basic biology and morphology.

C. albicans is polymorphic, capable of switching between unicellular yeast-forms and elongated pseudohyphae and hyphae. The yeast cells are round and separate readily, while pseudohyphae are elongated yeast that usually grow in a branching pattern, thought to

facilitate nutrient foraging. Lastly, hyphal cells are long and highly polarized, promoting inter-cell adhesion (BERMAN AND SUDBERY 2002). These phenotypes influence *C. albicans* interactions with host immune system cells, such as neutrophils and macrophages, and affect formation of biofilms, which are complex microbial communities attached to surfaces (LO *et al.* 1997; RICHARD *et al.* 2005). In addition to cell morphology, other attributes that contribute to *C. albicans* success as a commensal and pathogen are its nutritional versatility and resistance to oxidative stress, enabling growth in different host niches (FLECK *et al.* 2011; DUNKEL *et al.* 2013).

Sufficient nutrient acquisition is essential for the onset and development of infections by microbial pathogens. *C. albicans* has specifically adapted to the nutritional conditions supplied by the host (BROCK 2009). In contrast to its generally nonpathogenic relative *Saccharomyces cerevisiae, C. albicans* can use proteins as a nitrogen source by secreting aspartic proteases (NAGLIK *et al.* 2003; DUNKEL *et al.* 2013). Additionally, *C. albicans* regulates carbon source utilization in a distinct manner, and unlike other fungi, can make use of both preferred and nonpreferred carbon sources simultaneously instead of solely switching to the utilization of the more favorable carbon source (SANDAI *et al.* 2012). One possibility for this unique carbon catabolite control is that it allows *C. albicans* to transition from one niche to another without the lengthy growth pauses associated with switching carbon sources (LORENZ 2013). These examples demonstrate the importance of metabolism to virulence. The rest of this dissertation will describe another aspect of *C. albicans* virulence involving the role of sulfur metabolism in resistance to oxidative stress caused by reactive sulfur-containing molecules.

1.2 Oxidative stress in microbes

Oxidants released by microbial competitors and eukaryotic hosts quickly poison microbes. In response, adaptive strategies are activated involving scavenging enzymes, transport proteins, or repair systems. The following sections define and describe the ingredients of oxidative stress, and the resulting cellular responses.

1.2.1 Introduction to reactive oxygen, nitrogen, and sulfur species (ROS, RNS, RSS)

Oxidative stress refers to elevated intracellular levels of reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive sulfur species (RSS) that are capable of causing damage to lipids, proteins, and DNA (GILES AND JACOB 2002). Although these chemically reactive chemical species are often associated with oxidative stress, they are also involved in essential redox reactions occurring in all living organisms, especially serving as signaling molecules to regulate biological and physiological processes (FINKEL 2011; WANG 2012; ADAMS *et al.* 2015). Each type of reactive species exhibits unique properties.

ROS are reactive oxygen-containing molecules that are by-products of aerobic metabolism and include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH·). Hydrogen peroxide is generated from superoxide produced by mitochondria and NADPH oxidases (BRAND 2010). Superoxide accumulation is associated with oxidative stress by causing damage and inactivating proteins containing iron-sulfur clusters (FRIDOVICH 1997; BAEZ AND SHILOACH 2014). The 3rd type of ROS, the hydroxyl radical, indiscriminately oxidizes lipids, proteins, and DNA, resulting in damage and genomic instability (DIZDAROGLU AND JARUGA 2012). Similarly to ROS, RNS are also known to cause oxidative modification of proteins, lipids, and other pertinent biological molecules.

RNS are various nitric oxide (NO·)-derived compounds resulting from interactions of biologically generated free radicals, such as NO· with the superoxide anion, to form more reactive species resulting in multiple biological effects (PATEL *et al.* 1999). Examples of RNS include nitric oxide, peroxynitrite ($ONOO^-$), higher oxides of nitrogen such as nitrite (NO_2^-), and *S*-nitrosothiols (RSNOs). Nitric oxide, produced by NO synthases from L-arginine and oxygen or by nitrate reduction by anaerobic bacteria, is the main RNS produced by cells and is the main source for other RNS. RNS can react with lipids, DNA and RNA bases, metal co-factors, and proteins, leading to the inhibition of their activities (ZMIJEWSKI *et al.* 2005; SAWA AND OHSHIMA 2006; MARTINEZ AND ANDRIANTSITOHAINA 2009). Therefore, both RNS and ROS are known to modify relevant biological molecules. The same concept applies to other reactive species such as RSS, although the role of RSS in this context has been less studied. RSS differ significantly from ROS and RNS with regards to their diverse chemistry and oxidations states.

RSS are chemically reactive sulfur-containing molecules that can oxidize or reduce biomolecules under physiological conditions (GRUHLKE AND SLUSARENKO 2012). Recent studies of biological RSS predict that under physiological conditions sulfur can occur in approximately 10 different oxidation states ranging from -2, such as sulfides and thiols, to +6, such as sulfates. This allows for the occurrence of several relevant biological species with sulfur in higher oxidation states, some of which have yet to be characterized (GILES AND JACOB 2002). Examples of RSS include sulfite, sulfide, disulfide (RSSR), disulfide-S-oxides, and sulfenic acids (RSOH). Reactive sulfur species are formed as a result of sulfate (SO_4^{2-}) or sulfite (SO_3^{2-}) reduction, or as a byproduct of the reactions of physiologically relevant thiol-containing molecules such as cysteine and glutathione (BRANNAN 2010). RSS can also

be formed with relative ease under oxidative stress where cellular concentrations of peroxides and superoxide sufficient to form peroxynitrite, are also high enough to form RSS (RADI *et al.* 1991; GILES *et al.* 2001).

In general, RSS react specifically with thiols and their rate of reaction with proteins is predicted to depend on the degree of dissociation of the thiol. RSS such as disulfide-S-oxides inhibit enzymes by oxidizing thiol groups on cysteine residues causing the release of any bound zinc atoms or inactivation of essential catalytic cysteine residues. RSS inhibition of enzymes occurs in a non-competitive manner. RSS have also been shown to undergo thiolation reactions with cysteine residues resulting in the modification and subsequently the inhibition of a number of important enzymes such as alcohol dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase (GILES *et al.* 2002).Therefore, RSS will preferentially react at redox-sensitive cysteine residues, consequently causing RSS to be considered as oxidative stressors with their own cellular targets and redox-transformation pathways (SRINIVASAN *et al.* 1997; GILES *et al.* 2001). Oxidation of cellular thiols not only inhibits a number of redox proteins and enzymes, it also consumes glutathione (GSH), which is present in cells as a cellular redox-buffer that acts as a first line of defense to detoxify ROS (LUSHCHAK 2012). Sulfite reacts with glutathione to form S-sulfoglutathione, making glutathione a less readily available antioxidant. Other reactions include sulfite with cysteine to form *S*-sulfocysteine, an antagonist of glutamate dehydrogenase (OLNEY *et al.* 1975). Moreover, RSS such as sulfide inhibit aerobic respiration by inhibiting cytochrome c oxidase in the electron transport chain of mitochondria. Mitochondrial cytochrome c oxidase is inhibited by sulfide in a biphasic manner. The oxidized enzyme occurs in at least two forms, and one of these forms reacts aerobically with sulfide to form two intermediates

that are otherwise involved in the reaction of oxygen with the reduced enzyme. Sulfide directly reduces the oxygen-reactive a3CuB binuclear center of this enzyme (NICHOLLS *et al.* 2013). Although the wider biochemical implications of RSS formation are currently being explored, their interactions with other reactive species are also a topic of increasing interest.

Reactive sulfur species are formed by the oxidation of GSH in the presence of a variety of ROS, such as peroxides and peroxynitrite (FINLEY *et al.* 1981; BONINI AND AUGUSTO 2001; GILES *et al.* 2001). Additionally, the reaction of the ROS superoxide and the RNS nitric oxide generates another RNS, peroxynitrite, a potent stressor that damages DNA, lipids, and proteins (BUCHCZYK *et al.* 2000). Nitric oxide also reacts with cysteine thiols to form *S*-nitrosothiols, an effective inhibitor of fungi and Gram-positive bacteria (FINNEN *et al.* 2007; CARIELLO *et al.* 2012). Therefore, ROS, RNS, and RSS, are toxic individually and in combinations, increasing their physiological impacts and thusly, microbes have evolved several ways to protect against their toxicity.

1.2.1.1 Mechanisms of resistance to oxidative stress in microbes

Several of the ‘‘reactive species’’ mentioned in the previous section are released by phagocytes, such as macrophages and neutrophils, to combat pathogens. Examples of reactive species released by phagocytes include RNS nitric oxide, the ROS superoxide anion, and the RSS sulfite (CUNHA *et al.* 1993; MITSUHASHI *et al.* 1998; CRAIG AND SLAUCH 2009; PROLO *et al.* 2014). Additionally, the RSS sulfide and RNS nitrite and nitric oxide are released by gut microbes and play roles in cell signaling and inhibiting growth of other bacterial species (LINDEN *et al.* 2010; TISO AND SCHECHTER 2015; TOMASOVA *et al.* 2016).

Therefore, bacteria and fungi are constantly exposed to reactive species and have a variety of mechanisms of protection against oxidative stress caused by these chemicals.

Two types of defense mechanisms involve keeping the concentration of the reactive species at acceptable levels or repairing oxidative damages. Two superoxide dismutases (SOD), which convert O_2^- to H_2O_2 and O_2, have been described in the bacteria *Escherichia coli* (NIEDERHOFFER et al. 1990; COMPAN AND TOUATI 1993). Furthermore, the H_2O_2 produced in *E. coli* is removed by two catalases, hydroperoxidase I and hydroperoxidase II, yielding H_2O and O_2, as well as by glutathione peroxidase, which decomposes *in vitro* hydrogen peroxide in a glutathione-dependent manner (VON OSSOWSKI *et al.* 1991; ARENAS *et al.* 2010). Similar enzymes are present in *C. albicans* and other fungi. The secondary defense mechanisms for repairing oxidative damage include repair systems involving proteolytic and lipolytic enzymes, such proteinase complexes and phospholipases that degrade damaged proteins and phospholipids, respectively, so that their components can be reused (AHMAD 1995). Moreover, defense mechanisms also detoxify oxidized amino acids. For example, oxidation of methionine to methionine sulfoxide can be repaired by methionine sulfoxide reductases, which are essential for the pathogen *Salmonella Typhimurium* resistance to host-induced oxidative stress (DENKEL *et al.* 2011). Yeast lacking methionine sulfoxide reductase are also more sensitive to oxidative stress (MOSKOVITZ *et al.* 1997). Therefore, the mechanisms of oxidative stress resistance in fungi are like those in bacteria.

In addition to catalase detoxification of host-derived H_2O_2, it has been proposed that when fungal cells suffer oxidative stress, they generally form biofilms or adopt morphological forms that reduce the surface area exposed to the environment (LI *et al.* 2009). Fungi also utilize alternative respiration pathways during oxidative

phosphorylation inhibition by reactive species. For example, when Complex I of the mitochondrial electron transport chain is inhibited, fungi use an alternative NADH dehydrogenase to accept electrons from NADH and transfer them to ubiquinone directly. Unlike Complex I, however, this enzyme does not pump protons out of the matrix across the inner mitochondrial membrane, and the free energy between NADH and ubiquinone is lost as heat (LUTTIK *et al.* 1998; LI *et al.* 2009). The yeast *C. albicans* employs alternative respiration pathways to resist oxidative stress. The next section will describe this occurrence in more detail, as well as specific defense mechanisms used against RNS, ROS, and RSS.

1.2.1.2 *Candida albicans* resistance to oxidative stress

As mentioned previously, *C. albicans* inhabits a wide variety of niches located in its human host, and part of its success as a pathogen is due to its resistance to oxidative stress. Resistance to oxidative stress is mediated by several pathways involving membrane transporters and antioxidant enzymes. The two major oxidative stress signaling pathways are the Cap1 and Hog1 pathways, and the three major antioxidant systems include the catalase, glutathione, and thioredoxin systems (KOMALAPRIYA *et al.* 2015). Hog1 is a mitogen-activated protein kinase, mediating adaptation at both transcriptional and post-transcriptional levels, that is involved in several processes ranging from oxidative and osmotic stress responses, to cell wall biosynthesis and chlamydospore formation (ALONSO-MONGE *et al.* 2003; SMITH *et al.* 2004; ENJALBERT *et al.* 2006). *C. albicans* mutants with mutations in *HOG1* displayed an increased sensitivity to ROS, such as hydrogen peroxide or superoxide, as well as reduced virulence in mice and enhanced susceptibility to phagocytic cells (ALONSO-MONGE *et al.* 1999; ALONSO-MONGE *et al.* 2003). The 2nd major oxidative stress

pathway is mediated by Cap1, a transcription factor largely responsible for the activation of oxidative stress genes in *C. albicans* (ZNAIDI et al. 2009). Following exposure to H_2O_2, *C. albicans* induces the expression of all three antioxidant systems primarily via Cap1, and with some contribution from Hog1 signaling (ENJALBERT *et al.* 2006; KOMALAPRIYA *et al.* 2015). Adaptation to nitrosative and RSS stress involves distinct transcription factors and transporters.

Exposing *C. albicans* to nitric oxide induces upregulation of the *YHB1* gene, which encodes the flavohemoglobin Yhb1, a nitric oxide dioxygenase that confers protection by enzymatically converting NO to harmless nitrate (ULLMANN *et al.* 2004; HROMATKA *et al.* 2005). The transcription factor Cta4 is required to induce *YHB1* transcription during nitrosative stress, and deletion of either *CTA4* or *YHB1* confers growth sensitivity to the NO donor dipropylenetriamine NONOate, and a small but significant decrease of *C. albicans* virulence (HROMATKA *et al.* 2005; CHIRANAND *et al.* 2008).

In both *C. albicans* and *S. cerevisiae,* NO also induces the expression of a gene involved in RSS detoxification named *SSU1,* which encodes a sulfite transporter (HROMATKA *et al.* 2005; SARVER AND DERISI 2005). Studies in *S. cerevisiae* show that deletion of *SSU1* results in sulfite sensitivity, while its overexpression confers an increase in sulfite resistance (AVRAM AND BAKALINSKY 1997). The *S. cerevisiae* transcription factor Fzf1 mediates responses to both RNS and RSS (PARK *et al.* 1999; SARVER AND DERISI 2005). Both Fzf1 and Cta4 are required for *SSU1* induction by NO in the respective organisms (SARVER AND DERISI 2005; CHIRANAND *et al.* 2008). The *S. cerevisiae Δssu1* mutant shows increased NO sensitivity when grown in synthetic complete (SC) medium, but no change in NO sensitivity when tested in YEPD (rich) medium. One possibility is that NO rapidly converts to nitrite,

and Ssu1 may also have a function in exporting nitrite. However, the *C. albicans Δssu1* mutant shows no change in NO sensitivity compared to wild type, regardless of whether SC or YEPD media was used, though, the mutant is more sensitive to sulfite (CHIRANAND *et al.* 2008). Aside from the involvement of Ssu1 in sulfite resistance in *C. albicans,* Ssu1 also plays a role in cysteine tolerance.

C. albicans is sensitive to increasing levels of cysteine, which is present in the mammalian gut at considerable concentrations supporting various intestinal functions (WAIN *et al.* 1975; CIRCU AND AW 2011). *C. albicans* lacking *SSU1* are also sensitive to cysteine, due to the conversion of cysteine to sulfite by cysteine dioxygenase, Cdg1 (HENNICKE *et al.* 2013). The transcription factor Zcf2 is required for cysteine and sulfite resistance, as well as cysteine-inducible *CDG1* and *SSU1* expression. This thesis confirms and expands on these findings, as well as elucidates the role of Zcf2 in *C. albicans* resistance and adaptation to sulfite.

1.2.2 Hormesis: A little poison is good for you

Hormesis refers to enhanced fitness due to the exposure of cells to low doses of stress-inducing physiological treatments, such as toxic chemicals (MCCLURE *et al.* 2014). This adaptive response is seen in many organisms especially in regards to oxidative stress. For example, once cells have been treated with a low concentration of H_2O_2 they are more resistant to H_2O_2 killing at a lethal concentration. This occurrence has been observed in various microorganisms, from the bacteria *E. coli,* to fungi such as *S. cerevisiae, A. niger,* and

C. albicans (JAMIESON 1992; JAMIESON *et al.* 1996; CARMEL-HAREL AND STORZ 2000; LI *et al.* 2008b). The mechanism of this adaptation in *C. albicans* has been previously studied.

Given their toxic nature, the intracellular concentration of ROS in cells is normally regulated at very low levels by a series of antioxidant defenses. Unsurprisingly, the activities of antioxidant enzymes are observed to increase during adaptation. In *C. albicans*, catalase, superoxide dismutase, and glutathione peroxidase activity, involved in H_2O_2 detoxification, were elevated by a nonlethal dose of H_2O_2 or menadione, a chemical that induces ROS generation. The activity of catalase was much higher than glutathione peroxidase. Additionally, the activity of SOD increased significantly upon exposure to menadione compared with H_2O_2 treatment (JAMIESON *et al.* 1996). The adaptive response in *S. cerevisiae* and *A. niger* also includes cytosolic catalases, SOD, as well as various peroxidases (GODON *et al.* 1998; LI *et al.* 2008b).

Additionally, non-enzymatic antioxidants also play a role in H_2O_2 adaptation. For example, depletion of cellular glutathione suppressed the adaption to H_2O_2 in *S. cerevisiae,* and GSH concentrations in *A. niger* increased in response to H_2O_2 addition (IZAWA *et al.* 1995; LI *et al.* 2008b). Metabolic alteration also occurs as part of the adaptive stress response in *S. cerevisiae* and will be discussed in more detail later in this chapter. First, I will discuss the metabolic pathway that is most relevant in regards to the topic of this thesis regarding *C. albicans* adaptation to RSS.

1.3 Regulation of sulfur metabolism

Sulfur is a constituent of many organic molecules, including amino acids, the antioxidant glutathione, and Fe-S clusters that are required for the activity of many enzymes involved in redox reactions. Sulfur is therefore an essential element for living

organisms. Accordingly, the sulfate assimilation pathway is conserved among many kingdoms.

1.3.1 Sulfur metabolism pathways in bacteria, plants, and fungi

Bacteria, plants, and fungi all metabolize sulfur via a somewhat conserved pathway (THOMAS AND SURDIN-KERJAN 1997; SEKOWSKA *et al.* 2000; KOPRIVOVA AND KOPRIVA 2014). Microbial metabolism of sulfur compounds has led to the development of a biological sulfur cycle (Figure 1), which is complex due to the multiple oxidation states of sulfur (THOMAS AND SURDIN-KERJAN 1997). As shown in Figure 1, organisms use sulfur in a variety of ways: for biosynthesis, as a terminal electron acceptor in a respiratory system, or as an energy source.

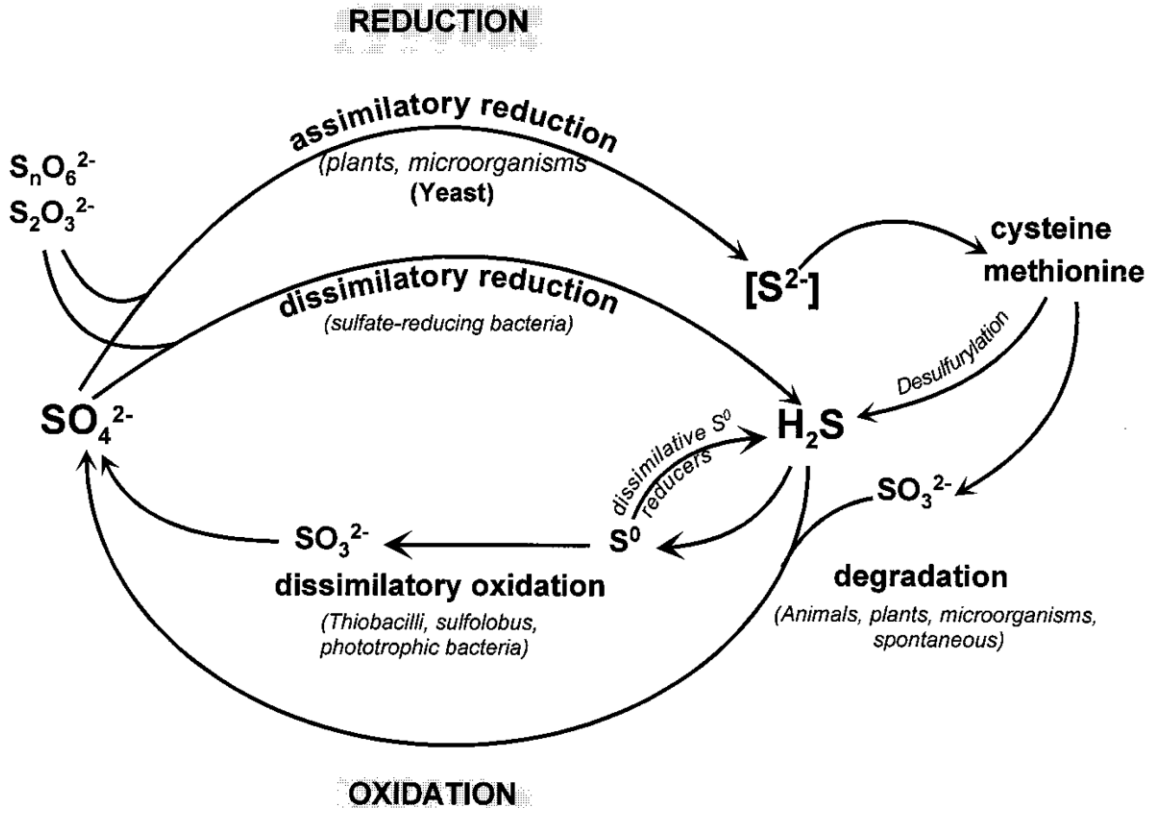

Figure 1-Biological sulfur cycle. *Note:* Reprinted from *Metabolism of Sulfur Amino Acids in Saccharomyces cerevisiae* (THOMAS AND SURDIN-KERJAN 1997).

In general, extracellular sulfate (SO_4^{2-}) is transported into the cell, where it is reduced to 5'-adenylylsulfate (APS), which is then phosphorylated to 3'-phospho-5'-adenylylsulfate (PAPS). PAPS is then reduced to sulfite and subsequently sulfide; both are reactive sulfur species that were discussed earlier in this chapter. Thus, RSS are also produced endogenously in organisms via sulfate assimilation, and in the case of *C. albicans,* little is known about how the endogenous toxicity of sulfite is avoided. Sulfide is then used to make amino acids such as cysteine and methionine. Figure 2 illustrates a simplified version of the sulfate assimilation pathway in the organism that is the focus of this thesis, *C. albicans.*

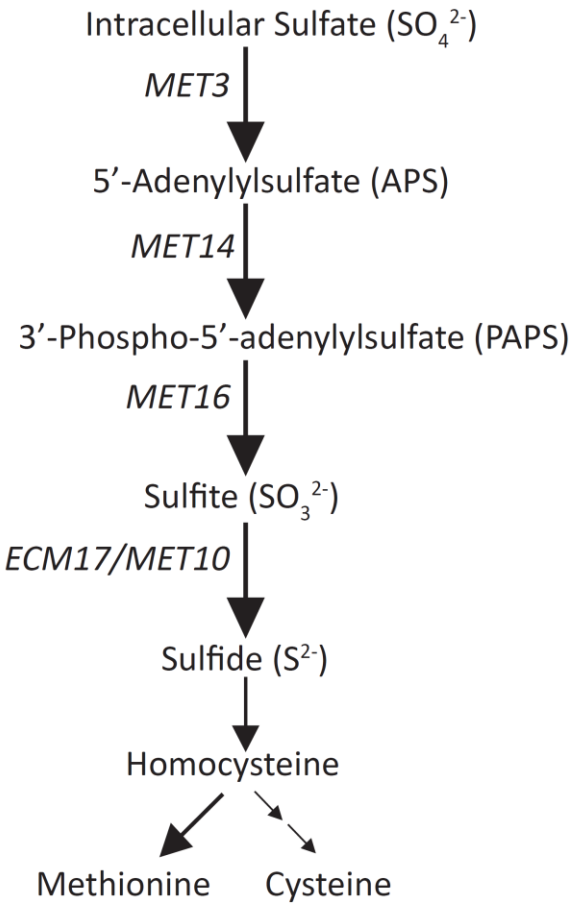

Figure 2- Sulfate assimilation pathway in *C. albicans*. *MET3,* ATP sulfurylase; *MET14,* APS kinase; *MET16*, PAPS reductase; *ECM17/MET10*, Sulfite reductase (β and α subunits).

1.3.2 Sulfite production and antimicrobial properties

As discussed earlier, sulfite is a metabolite produced in bacteria, fungi, and plants during reductive sulfate assimilation. In humans, sulfite is generated during oxidative catabolism of the sulfur-containing amino acid cysteine, and is also released by neutrophils in response to activation by bacterial lipopolysaccharides, suggesting that neutrophils release sulfite as an antimicrobial agent (MITSUHASHI *et al.* 1998; MITSUHASHI *et al.* 2002; STIPANUK *et al.* 2006). Sulfite is also used in the food industry as an antimicrobial and anti-browning agent; especially in wine preparation as a selective inhibitor of bacteria (TAYLOR

et al. 1986; RANGAN AND BARCELOUX 2009). There are several mechanisms for sulfite-induced toxicity.

One mechanism of sulfite toxicity in eukaryotic mitochondria is through inhibition of glutamate dehydrogenase, which leads to a decrease in ATP synthesis (ZHANG *et al.* 2004). In *S. cerevisiae*, sulfite toxicity involves the inhibition of alcoholic fermentation via glyceraldehyde-3-phosphate inhibition, which also leads to a rapid and drastic decrease of ATP concentration in cells (HINZE AND HOLZER 1985). Likewise, in *C. albicans,* growth in the presence of sodium sulfite under acidic conditions caused a decrease in ATP concentrations, as well as inhibition of ethanol production. These results indicated that the candidacidal/candidastatic effect of sodium sulfite was due to disruption of alcohol fermentation and aerobic respiration (OGASAWARA *et al.* 2008).

1.3.3 Role of metabolic pathways in oxidative stress resistance

In some instances, alteration of metabolic pathways occurs as a mechanism to protect against oxidative stress. For example, the thioredoxin system is one of the three major antioxidant systems in yeasts that detoxifies H_2O_2 via peroxiredoxin, which uses the reductant thioredoxin. Oxidised thioredoxin is then reduced using NADPH via thioredoxin reductase (PANNALA AND DASH 2015). NADPH is thus considered an important compound that provides reducing power for such antioxidant reactions, and in general, the pentose phosphate pathway (PPP) is considered the major production site of NADPH. Accordingly, in response to oxidative stress, *S. cerevisiae* reroutes metabolic flux to the PPP pathway and PPP enzyme mutants have an increased susceptibility to H_2O_2, suggesting that *S. cerevisiae* adapts to oxidative stress by diverting carbon sources from glycolysis to PPP to produce sufficient amounts of NADPH (JUHNKE *et al.* 1996; RALSER *et al.* 2007). However, this

suggestion has not been directly tested because those studies do not consider possible changes in overall carbon flux during oxidative stress. Contrastingly, when the fungus *A. niger* was grown in the presence of H_2O_2, glucose uptake rates fell and intracellular NADPH levels decreased (Li *et al.* 2008a). Therefore, experiments involving the use of isotopically labelled carbon sources are needed to provide new insight. In these examples, oxidative stress by hydrogen peroxide altered glucose metabolism, however, other studies have shown that the opposite situation occurs as well, where altering metabolism affects resistance to stress.

In *C. albicans* particularly, there have been several reports where growth on physiologically relevant carbon sources induced fungal cell wall remodeling and modulation of the cell wall proteome and secretome, thus affecting important virulence parameters such as stress and drug resistance, adherence, biofilm formation, and infection outcome (Ene *et al.* 2012a; Ene *et al.* 2012b; Ene *et al.* 2013). For example, cells grown on physiological levels of lactate are more resistant to osmotic stress in a Hog-independent manner. Lactate is a nonfermentable carbon source that is found in various anatomical niches. Lactate-grown cells are also more resistant than glucose-grown cells to antifungal drugs such as caspofungin, tunicamycin, and amphotericin B, all of which interact with components of the *C. albicans* cell walls. These observed phenotypes extend to other pathogenic *Candida* species and other physiological carbon sources (Ene *et al.* 2012a). The type of carbon source utilized by *C. albicans* also affects interactions with the host immune system. For example, lactate-grown *C. albicans* cells are rendered less visible to the immune system and are taken up by macrophages less efficiently than glucose-grown cells (Ene *et*

al. 2013). In this thesis, I describe how inducing an increase in sulfate assimilation pathway flux causes *C. albicans* to be more resistant to the RSS sulfite.

1.4 Zinc cluster family transcription factors

C. albicans requires coordinated expression and regulation of genes, especially because of its dimorphic life style and the distinct niches it occupies. Transcription factors (TF) play critical roles in cell functions and responses to different environments. The *C. albicans* genome encodes ~240 TFs, comprising about 4% of *C. albicans* transcripts (HOMANN *et al.* 2009). Therefore, TFs make up the single largest family of proteins and coordinate essential functions including biofilm formation, drug resistance, and dimorphism (LIU 2001; COWEN *et al.* 2002; NOBILE *et al.* 2009). Interestingly, zinc-finger transcription factors are enriched in pathogenic *Candida* species and show enhanced rates of evolution, indicating key roles in recent adaptation (BUTLER *et al.* 2009).

The Zinc Cluster Family (ZCF), also known as zinc binuclear cluster proteins, consists of $Zn(II)_2Cys_6$ DNA-binding proteins that are restricted to the fungal kingdom; *C. albicans* has 82 ZCFs (SCHILLIG AND MORSCHHAUSER 2013). A subset of 35 ZCFs are expanded through duplication and diversification in pathogenic fungi, and are missing from rare pathogens and nonpathogenic yeasts (BUTLER *et al.* 2009; ISSI *et al.* 2017). The ZCF TFs are characterized by the well-conserved motif $CX_2CX_6CX_{5\text{-}12}CX_2CX_{6\text{-}8}C$ in their DNA-binding domain. The most studied member of this family is *S. cerevisiae* Gal4, a transcriptional activator of genes involved in galactose metabolism to glucose (MARMORSTEIN *et al.* 1992). The *S. cerevisiae* genome encodes 55 proteins with the Zn_2Cys_6 motif, making this TF one of the largest in yeast, and regulating diverse cellular processes such as sugar and amino acid metabolism, as well as stress responses (MACPHERSON *et al.* 2006). Several large-scale

phenotypic characterizations have demonstrated the importance of ZCFs, however, the specific functions of most family members remain unknown and must be evaluated one at a time (HOMANN *et al.* 2009; VANDEPUTTE *et al.* 2011; BOHM *et al.* 2016).

Two ZCF TFs with roles in virulence have been recently identified in *C. albicans*. Knockout mutants of both *ZCF15* and *ZCF29* are hypersensitive to ROS, and transcriptomic analysis of the mutants under standard growth conditions revealed a large network of target genes that control macro and micronutrient homeostasis. Both TFs also regulate a separate set of genes involved in ROS detoxification and ribosome biogenesis down-regulation (ISSI *et al.* 2017). Another ZCF TF, Zcf21, is involved in setting the default state of low expression of multiple cell wall components, preventing *C. albicans* self-aggregation and assembling a cell surface configuration suitable for colonizing mammalian tissues and evading immune surveillance (BOHM *et al.* 2016). Two ZCF TFs of relevance were mentioned earlier, Cta4 and Zcf2, implicated in RNS and RSS, respectively. The rest of the thesis will explore the role of Zcf2 in *C. albicans* adaptation to sulfite, as well as the transcriptome this transcription factor controls.

Chapter 2: Materials and Methods

2.1 Strains and growth conditions

Candida albicans strains used in this study are listed in Appendix A. The strain termed 'Wild type' is *Δarg4* and is derived from Homann *et al*. All deletion mutants were confirmed by PCR-based analysis. *C. albicans* strains were cultured from frozen stocks onto yeast extract-peptone-dextrose (YEPD) medium agar plates supplemented with uridine

and adenine (1% yeast extract, 2% peptone, 2% dextrose, 2% agar, 40 mg/ml uridine, and 20mg/ml adenine, pH ~6.0).

2.1.1 General propagation

C. albicans strains were generally propagated at 30°C on a spinning tube-rotator in liquid YEPD medium or SD medium (Yeast Nitrogen Base: without amino acids or carbohydrate and with ammonium sulfate (YNB) from US Biological, 2% glucose, 80 mg/L arginine).

2.1.2 Analyzing growth in media containing various sulfur sources

Overnight cultures of *C. albicans* wild type and *Δzcf2* mutant strains were washed twice in water, resuspended in water, and incubated at 30°C for 1.5 hours. Following incubation, cells were diluted to an OD_{600} of 0.1 in sulfur-free media supplemented with 2.5 mM of various sulfur sources (sodium sulfate, sodium sulfite, methionine, cysteine, taurine, sodium thiocyanate, sodium taurocholate hydrate, sodium isethionate, sodium thiosulfate, sulfoacetate, potassium methanesulfonate, or no sulfur). The sulfur-free media was made as described by Florian M. Freimoser (http://www.openwetware.org/index.php?title=Freimoser:Recipes:Yeast:SC_Sfree&oldid=9632) and contains the following: 4 mM magnesium chloride, 1.7 mM sodium chloride, 1 mM calcium chloride dihydrate, 8 µM boric acid, 0.25 µM copper chloride dihydrate, 0.6 µM potassium iodide, 2 µM zinc acetate, 2 µM manganese chloride tetrahydrate, 1 µM sodium molybdate, 1.5 µM ferric chloride hexahydrate, 37.8 mM ammonium chloride, 7.5 mM potassium dihydrogen phosphate, vitamin solution, 100 mg/L arginine and 2 % glucose. Subsequently, the cells were incubated for 21 hours at 30°C in sterile glass tubes placed in

a spinning rotator. End-point readings of each culture were taken manually using a spectrophotometer.

2.1.3 Analyzing growth in nitrogen poor media

Overnight cultures of wild type-Arg$^+$ and *Δzcf2*- Arg$^+$ strains were washed twice in water, resuspended in water, transferred to sterile glass tubes, and incubated for 1 hour in a spinning rotator at 30°C. Cells were then centrifuged and resuspended in YCB media (BD DIFCO™) supplemented with various concentrations of ammonium chloride (NH_4Cl): 0, 0.25, 0.5, 2.5, 5, and 10 mM, or various nitrogen sources: glutamic acid, ammonia acetate, and urea (1%). Subsequently, the cells were transferred to a 48-well plate (500 µl/well) and incubated overnight (16 hours) with continuous shaking at 30°C in a plate reader (Tecan Infinite 200 pro). OD_{600} readings were taken every 30 minutes.

2.1.4 Testing for methionine auxotrophy

Overnight cultures were pelleted, washed once in water, resuspended in water, and incubated in sterile glass tubes placed inside a spinning rotator for 45 minutes at 30°C. Cells were then diluted to OD_{600} 0.03 in SD medium with or without 0.75 mg/ml methionine, and transferred to a 48-well plate, (500 µl/well) and grown overnight (16 hours) at 30°C in a plate reader with continuous shaking. OD_{600} readings were taken every 30 minutes.

2.2 Analyzing sulfite and sulfide toxicity in various media

For sulfite and sulfide growth assays, overnight cultures were diluted in YEPD pH 4.0 to an optical density (OD_{600}) of 0.03, transferred to 48-well plates (500 µl/well) and grown for 3 hours at 30°C in a plate reader with continuous shaking. Cells were then challenged with various amounts of sulfite, Na_2SO_3, (0, 4, 8, or 12 mM) or sulfide, Na_2S-

$9H_2O$, (0, 1, or 2 mM) from freshly prepared stock solutions, and incubated at 30°C with continuous shaking overnight (16 hours). OD_{600} readings were taken every 30 minutes.

2.3 Adaptation assays: Adapting *Candida albicans* to sulfite

2.3.1 Finding pretreatment concentrations

Concentrations of sulfite, sulfide, and nitrite needed to pretreat *C. albicans* against sulfite were determined by diluting overnight cultures to an OD_{600} of 0.03 in YEPD pH4, and growing the cells for 3 hours at 30°C in a 48-well plate (500 µl/well) with continuous shaking in a plate reader. After the 3 hours, sulfite, sulfide, and nitrite ($NaNO_2$) were added at various concentrations ranging from 0 to 1 mM, and then incubated in the plate reader overnight (16 hours) at 30°C, taking readings every 30 minutes. The highest concentration that did not inhibit growth was the sub-toxic concentration used for pretreatment assays.

2.3.2 Adaptation to sulfite following pretreatment with sulfite, sulfide, or nitrite.

Overnight cultures were diluted in YEPD pH 4.0 to an initial OD_{600} of 0.03, transferred to a 48-well plate, and grown for 3 hours at 30°C in the plate reader with continuous shaking. Cultures were then pre-treated with 0 mM, 0.25 mM sulfite, 0.012 mM sulfide, or 0.5 mM nitrite from freshly prepared stock solutions and grown at 30°C with shaking for an additional 15-20 minutes. Following pre-treatment, cultures were challenged with various concentrations of sulfite (0, 2, 4, or 8 mM), and grown overnight (16 hours) at 30°C with continuous shaking in a plate reader. OD_{600} readings were taken every 30 minutes.

2.3.3 Analyzing kinetics of sulfite adaptation by varying pretreatment times

Overnight cultures were diluted in YEPD pH 4.0 to an initial OD_{600} of 0.03, transferred to a 48-well plate, and grown for 3 hours at 30°C in the plate reader with continuous shaking. Cultures were then pre-treated with 0 mM, 0.25 mM sulfite or 0.012mM sulfide, from freshly prepared stock solutions. Subsequently, the cells were grown at 30°C with shaking for various times: 1, 2.5, 5, 7.5, 10, 15, and 20 minutes. Following pre-treatment, cultures were challenged with 0 and 6 mM of sulfite, and grown overnight (16 hours) at 30°C with continuous shaking in a plate reader. OD_{600} readings were taken every 30 minutes.

2.3.4 Finding minimum amount of sulfite and sulfide needed to pre-adapt *Candida albicans* to sulfite

Overnight cultures were diluted in YEPD pH 4.0 to an initial OD_{600} of 0.03, transferred to a 48-well plate, and grown for 3 hours at 30°C in the plate reader with continuous shaking. Cultures were then pre-treated with various concentrations of sulfite (0, 0.015, 0.031, 0.0625 mM) or sulfide (0, 0.003, 0.006, and 0.012 mM) from freshly prepared stock solutions and grown at 30°C with shaking for 20 minutes. Following pre-treatment, cultures were challenged with 0 and 2 mM of sulfite, and grown overnight (16 hours) at 30°C with continuous shaking in a plate reader. OD_{600} readings were taken every 30 minutes.

2.4 Construction of gene deletions

Homozygous deletion mutants of orf19.3120 were constructed in strain SN152 using methods previously described (NOBLE AND JOHNSON 2005). The ORF of each orf19.3120 allele was replaced with cassettes containing *Candida maltosa LEU2* and

Candida dubliniensis HIS1, whose 5'UTRs and ORFs were PCR amplified from plasmids pSN40 and pSN52, respectively.

Homozygous double deletion mutants of orf19.3120 and *SSU1* were constructed by deleting both copies of the *SSU1* allele from the orf19.3120 mutant using methods previously described with some variation (NOBLE AND JOHNSON 2005). The ORF of each *SSU1* allele was replaced with cassettes containing *C. dubliniensis ARG4* and *C. albicans* HygB, whose 5'UTRs and ORFs were PCR amplified from plasmids pSN69 and pAU34-CaHygB, respectively (BASSO *et al.* 2010).

Homozygous deletion mutants of *AMO1* were constructed in strain SN152 using methods previously described (NOBLE AND JOHNSON 2005). The ORF of each *AMO1* allele was replaced with cassettes containing *Candida maltosa LEU2* and *Candida dubliniensis HIS1,* whose 5'UTRs and ORFs were PCR amplified from plasmids pSN40 and pSN52, respectively.

Homozygous deletion mutants of *MET16* were constructed in strains SN152 and *Δecm17* using methods previously described with some variation (NOBLE AND JOHNSON 2005). The ORF of each *MET16* allele was replaced with cassettes containing *C. dubliniensis ARG4* and *C. albicans* HygB, whose 5'UTRs and ORFs were PCR amplified from plasmids pSN69 and pAU34-CaHygB, respectively (BASSO *et al.* 2010).

2.5 Construction of transcriptional and translational gene fusions

2.5.1 *ZCF2-GFP*

Tagging *ZCF2* at the N-terminus: Gibson Assembly® (New England BioLabs) was used to generate a plasmid containing approximately 1 kilobase (kb) of the *ZCF2* promoter region, followed by the *GFP* gene (729 nucleotides), and subsequently the *ZCF2* ORF and

3'UTR (~2.6 kb). The 3'UTR consists of 350 nucleotides downstream of the *ZCF2* ORF stop codon, and was amplified as one continuous fragment with the *ZCF2* ORF. The *ZCF2* promoter region and *ZCF2* ORF and 3'UTR were amplified from wild type genomic DNA, and the *GFP* gene was amplified from the CIp20_*ACT1-GFP*_3'UTR plasmid. The 5.6 kb vector fragment was amplified from the CIp10-*NAT* plasmid and included the *RPS1* and Nourseothricin resistance gene, *NAT.* The generated $ZCF2_{promoter}$-*GFP*-$ZCF2_{ORF}$-3'UTR containing plasmid was digested at the *StuI* restriction site, and transformed at the *RPS1* locus in a *C. albicans Δzcf2* mutant strain.

Tagging *ZCF2* at the C-terminus: The last 465 nucleotides of the *ZCF2* ORF minus the stop codon, as well as a 474 nucleotide-long *ZCF2* 3'UTR were inserted at regions flanking the *GFP* and *NAT* genes in the pCR2.1_*GFP-NAT* plasmid. *ApaI* and *BsaBI* restriction sites were introduced by amplification at the 5' and 3'end, respectively, of the *ZCF2* ORF region. Likewise, *SacI* and *KpnI* restriction sites were introduced at the 5' and 3'end, respectively, of the *ZCF2* 3'UTR. The two fragments were subcloned at those restriction sites in the pCR2.1_*GFP-NAT* plasmid. The $ZCF2_{end\ of\ ORF\ fragment}$-*GFP-NAT*-$ZCF2_{3'UTR}$ construct was amplified from the generated plasmid, and transformed in a *C. albicans ZCF2* heterozygote strain. All DNA amplifications were performed using OneTaq® DNA Polymerase (New England BioLabs).

2.5.2 *ZCF2-3xFLAG*

The plasmid containing the *ZCF2-3xFLAG* construct, where the *3xFLAG* tag was placed at the N-terminus, was created using Gibson Assembly®. The *ZCF2* promoter region (~1 kb) and *ZCF2* ORF and 3'UTR (~2.6 kb) were amplified using OneTaq® DNA Polymerase from wild type *C. albicans* genomic DNA. The *3xFLAG* tag was amplified using

Takara Ex Taq DNA Polymerase from a *C. albicans* strain previously transformed with a *CTA4-3xFLAG* construct. The 5.6 kb vector fragment, containing the *RPS1* and *NAT* genes, was amplified from the CIp10-*NAT* plasmid between the *Kpn1* and *EcoRI* restriction sites using the Phusion® DNA Polymerase (New England BioLabs).

The plasmid containing the *ZCF2-3XFLAG-Hyperactive* construct was created similarly to the plasmid containing the *ZCF2-3xFLAG* construct with the exception that all the fragments were amplified using the Takara Ex Taq DNA Polymerase. Both plasmids were digested at the *StuI* restriction site, and transformed at the the *RPS1* locus in a *C. albicans Δzcf2* mutant strain.

2.53 *SSU1-gLUC, MET3-gLUC*

SSU1-gLUC and *ACT1-gLUC* plasmids were a modification of an *URA3*-marker containing plasmid made by Anna Tillman. The *NAT* gene from CIp10-*NAT* plasmid was inserted between *Bst*BI and *SacI* restriction sites in place of the *URA3* marker. The luciferase reporter is a synthetic version of the *Gaussia princeps* luciferase gene fused to *C. albicans PGA59*, which encodes a cell wall protein. Luciferase expressed from this fusion translocates to the cell surface, allowing for detection of luciferase luminescence by adding coelenterazine to intact cells (ENJALBERT *et al.* 2009).

The *MET3-gLUC* plasmid was created by inserting 1 kb of the *MET3* promoter region between *XhoI* and *HindIII* restriction sites in place of the 1 kb *SSU1* promoter in the *SSU1-gLUC* plasmid. The plasmids were targeted to the *RPS1* locus by digesting *RPS1* at the *StuI* site on the plasmid and then transforming the linearized plasmid into *C. albicans* strains by electroporation.

2.6 Using luciferase assays to analyze *SSU1* and *MET3* induction

2.6.1 *SSU1-gLUC* induction in wild type, *Δzcf2, Δecm17, Δmet16,* or *Δecm17met16* in response to sulfite, sulfide, nitrite, cysteine, methionine, or cyanide.

Overnight cultures of the reporter strains were diluted in YEPD pH 4 to an OD_{600} of 0.25 in sterile glass culture tubes and grown at 30°C in a spinning tube-rotator to log phase for 4 hours. 1.0 mM sulfite, 0.5 mM sulfide, or 0.25 mM cyanide were added to the strains and grown for an additional 30 minutes, 0.5 mM nitrite was added to the strains and grown for 45 minutes. For testing cysteine induction, overnight cultures of the reporter strains were diluted in SD medium pH 4.8 and grown for 4 hours before adding 1.25 mM cysteine, and subsequently growing the cells for an additional hour.

Luciferase assay- After cells were grown as reported, they were collected by centrifugation, washed, and resuspended in luciferase assay buffer (LA buffer) as previously described (ENJALBERT *et al.* 2009). The cells were deposited in solid white multiwell plates (100 µl/well). 100 µl Coelenterazine (Promega, Madison, WI) in LA buffer was added to the cells in the solid white plate to a final concentration of 1.25 µl, and luciferase activity luminescence was recorded using a luminometer (Tecan).

2.6.2 *SSU1-gLUC* or *MET3-gLUC* induction in wild type, *Δzcf2, Δmet16,* or *Δmet32,* in response to increasing sulfate assimilation pathway flux

Reporter strains were diluted to an OD_{600} 0.25 in sterile glass culture tubes and grown at 30°C in a spinning tube-rotator to log phase for 3 hours in YEPD pH 4 medium. The cells were subsequently washed in water and grown at 30°C for 1 hour in defined SD medium without ammonium sulfate, containing or lacking 1% glucose, 10 mM ammonium

chloride, or 0.5 mM methionine. The cells were then analyzed for luminescence using the luciferase assay described above.

2.7 Constructing mutants in putative *Zcf2* and *Cta4* binding sites (motifs) in the *SSU1* promoter

2.7.1 Finding motifs using MEME

In order to find enriched motifs and thus a possible Zcf2 binding sequence, 1-kilobase promoter regions of Zcf2-dependent and independent genes, determined from the microarray experiment, were analyzed with the program MEME (BAILEY AND ELKAN 1994) (http://meme-suite.org/). 1 kb upstream regions of seven Zcf2-dependent genes were analyzed against a background of 1000 1kb upstream regions of Zcf2-independent genes.

2.7.2 Introducing motif mutations in *SSU1* promoter of *SSU1-gLUC* using site-directed mutagenesis by overlap extension polymerase chain reaction.

Primers used in constructing mutant strains are listed in Appendix B. Using site-directed mutagenesis by overlap extension as previously described (HO *et al.* 1989), substitution mutations were introduced in the *SSU1* promoter located in the plasmid containing the *SSU1p-gLUC* construct. First, two PCR reactions were carried out to amplify a region of the *SSU1* promoter and *gLUC* DNA on either side of the intended mutation, with the substituted nucleotides on a linker segment of the internal primers. Secondly, the two PCR products were fused in a third PCR reaction, and the resulting fusion product was digested with the enzymes *KpnI* and *BstXI* and ligated in the place of the native *SSU1* promoter in the plasmid containing the *SSU1p-gLUC* construct. The plasmids were confirmed by sequencing and transformed into *C. albicans* strain as previously described and targeted to the *RPS1* locus.

2.8 Transcript profiling to find sulfite-induced genes in wild type and *Δzcf2* mutant strains

2.8.1 Growth conditions

Two overnight cultures of each wild type and *Δzcf2* mutant strains were diluted in to an OD_{600} of 0.03 in 50 mL YEPD pH 4 medium and grown for 3 hours in flasks with shaking at 30°C. The cultures were then split into 25 mL aliquots and sulfite (0 mM or 0.25 mM) was added. Subsequently, cultures were incubated at 30°C for 15 minutes with continuous shaking. The cultures were chilled on ice and pelleted by centrifugation. Finally, the pellets were flash frozen in liquid nitrogen and stored at -80°C.

2.8.2 RNA Isolation

Total RNA was isolated from frozen pellets by phenol extraction (SCHMITT *et al.* 1990) except that the RNA from the aqueous phase of the phenol extraction step was purified according to the Zymo Research Quick-RNA Miniprep protocol. Total RNA samples were eluted in RNase-free water and frozen at -80°C. The samples were sent to the Genomic and RNA Profiling Core at Baylor College of Medicine, analyzed for quality using the Agilent 2100 Bioanalyzer, and then processed as described:

2.8.3 Microarray hybridization

RNA was reverse transcribed to Cy3 (green) and Cy5 (red) fluorescently-labeled cDNA, and then hybridized to custom-designed oligonucleotide microarrays printed by Agilent Technologies (AMADID # 20166), previously described (NOBILE *et al.* 2012). cDNA from sulfite-treated wild type cells was hybridized against differentially labelled cDNA from non-treated wild type cells. Similarly, cDNA from sulfite-treated *Δzcf2* cells was hybridized against differentially labeled cDNA from non-treated *Δzcf2* cells.

2.8.4 Data processing

The $\log_2$ ratios of red versus green fluorescence were obtained from averages of 2 biological and 2 technical replicates in which the fluorescent dyes were swapped. Statistical significance was calculated through a standard T-test of (n=8). The coefficient of determination (R^2) was calculated by plotting replicates against each other; values above 0.9 show that the data are generally consistent:

- Wild type sulfite-treated green replicates 2 vs. 1, R^2=0.995;
- Wild type sulfite-treated red replicates 2 vs. 1, R^2=0.9957;
- Wild type non-treated green replicates 2 vs. 1, R^2=0.9904;
- Wild type non-treated red replicates 2 vs. 1, R^2=0.9921;
- *Δzcf2* sulfite-treated green replicates 2 vs. 1, R^2=0.9965;
- *Δzcf2* sulfite-treated red replicates 2 vs. 1, R^2=0.9955;
- *Δzcf2* non-treated green replicates 2 vs. 1, R^2=0.9926;
- *Δzcf2* non-treated red replicates 2 vs. 1, R^2=0.997.

2.9 Sulfide and sulfite measurement assays

2.9.1 Using BiBAGY agar to analyze hydrogen sulfide formation

Sulfite production was evaluated using BiBAGY agar containing bismuth that will combine with sulfite to form a black (Bi_2S_3) pigment (RIKKERINK *et al.* 1988). Overnight cultures were washed in water, serially diluted, spotted onto BiBAGY agar, incubated at 30°C for 48 hours, and visually assessed for color.

2.9.2 Using Sulfite Assay Kit to measure non-enzymatic sulfide to sulfite conversion

Sulfite concentrations were measured using MaxSignal® Sulfite Assay Kit (Bioo Scientific). Sulfite concentrations were measured in water and YEPD pH 4 media containing various amounts of sulfide or sulfite. All samples were incubated at 30°C for 30 minutes prior to being assayed for sulfite.

2.10 Analysis of ammonia release using an acid trap

Ammonia release by *C. albicans* wild type-*Arg*$^+$ and *Δzcf2-Arg*$^+$ mutant cells was assessed by using acid traps as previously described (PALKOVA *et al*. 1997; VYLKOVA *et al*. 2011). Briefly, overnight cultures of wild type-*Arg*$^+$ and *Δzcf2-Arg*$^+$ mutant cells were washed in water, diluted to an OD_{600} of 1.0, and spotted on 'GM-BCP' agar plates containing the pH indicator bromocresol purple: YNB without ammonium sulfate, 0.5 % allantoin, 1% casamino acids, and 0.1% Bromocresol purple, 2% agar, and 0.125 mM sulfite, determined as the highest subtoxic concentration, pH 4. To make the plates, a 2X solution of the media described above (minus the agar) was filter sterilized, warmed in a 65°C water bath, and diluted with 4% autoclave-sterilized agar solution. Subsequently, the spots were left to dry and reservoirs (microcentrifuge tube caps) containing 10% citric acid (150 µl/ cap) were placed underneath the colonies. Cells were incubated at 37°C and 20 µl samples from the acid traps were collected at 24, 48, and 72 hours, and stored at -20 °C. Parts per million (ppm) concentrations of ammonia were calculated based on a standard curve derived using the following method: Using a 100-ppm ammonia stock solution, a series of ammonia standard dilutions, ranging from 0 to 30 ppm, were created in 200 µl final volumes. To quantify ammonia, 800 µl Nessler's reagent was added to each standard dilution and the mixtures were then incubated for 30 minutes in the dark. OD_{400} readings were then taken using a spectrophotometer.

The frozen 20 µl samples were diluted 1/10 (in a final volume of 200 µl), and treated the same way as the standard dilutions used to make the standard curve. The experiment was also scaled down to be used with 96-well plates and a plate reader.

2.11 Using ChiP-exo to identify Zcf2 and Cta4 binding sites

Two overnight cultures of the *C. albicans ZCF2-3xFLAG* strain were each diluted in 2.0 liter of YEPD pH4 to an OD_{600} 0.08, and then incubated for 3 hours at 30°C with continuous shaking. The 2.0 liter cultures were then split into 1.0 liter aliquots, and 0.3 mM sulfite was added to one of each of the aliquots. Subsequently, the cells were incubated for 15 minutes at 30°C, fixed with 1% formaldehyde, and incubated for another 15 minutes at 25°C. Following fixation, 0.15 M glycine was added, the cells were incubated for an additional 5 minutes at 25°C, and then pelleted. OD_{600} measurements were used to obtain $1x10^9$ cells/sample. The pellets were washed twice in ST buffer containing protease inhibitors (10 mM Tris-HCl pH 7.5, 100 mM NaCl, and SigmaFAST™ protease inhibitor tablets from Sigma-Aldrich), and subsequently frozen in liquid nitrogen and stored at -80°C.

Similarly, two overnight cultures of the *C. albicans CTA4-9xMYC* strain were each diluted in 0.5 liters of YEPD, buffered with 80 mM HEPES to a pH of 7.5, to an OD_{600} 0.15, and then incubated for approximately 4 hours at 30°C with continuous shaking, or until an OD_{600} of 1.0 was reached. The 0.5 liter cultures were then split into 250 mL aliquots, 1.0 mM DPTA NONOate in 10 mM NaOH was added to one of each of the aliquots, and an equal volume of 10 mM NaOH was added to the control cultures. Subsequently, the cells were incubated for 10 minutes at 30°C, fixed with 1% formaldehyde, and incubated for another 15 minutes at 25°C. Following fixation, 0.15 M glycine was added, the cells were incubated

for an additional 5 minutes at 25°C, and then pelleted. OD_{600} measurements were used to obtain $1x10^9$ cells/sample. The pellets were washed twice in ST buffer containing protease inhibitors, and subsequently frozen in liquid nitrogen and stored at -80°C.

The frozen pellets were sent to Peconic in Pennsylvania, USA, for chromatin fragmentation, performance of the ChIP-exo assay, next-generation sequencing, and standard bioinformatics analysis.

2.12 Statistical analyses

Results of experiments are averages of three or more biological replicates and repeated several times unless otherwise noted. Statistics were calculated using GraphPad Software and significance was determined using unpaired *t* tests or r^2 -coefficients of determination (page 29).

Chapter 3: *Candida albicans* can grow on a variety of sulfur sources

C. albicans lives within several metabolic niches in its host environment and therefore must be able to acquire and metabolize a variety of nitrogen, sulfur, and carbon sources. Although *C. albicans* growth on various nitrogen and carbon sources has been previously examined, little is known about growth on various sulfur sources (ENE *et al.* 2012a; RAMACHANDRA *et al.* 2014). Therefore, the ability of *C. albicans* to grow on a variety of sulfur sources was examined, as well the involvement of the transcription factor Zcf2. Zcf2 became our focus when we screened a collection of transcription factor deletion mutants

from Homann *et al.* for increased sensitivity to sulfite (HOMANN *et al.* 2009). Because *Δzcf2* is sensitive to sulfite, a toxic metabolite produced during sulfate assimilation, we tested to see if this sensitivity was due to a defect in growth on different sulfur sources. When wild type and *Δzcf2* mutant strains were grown on a variety of sulfur sources, however, no difference between them was observed, except that *Δzcf2* grew less that wild type on sulfite and cysteine (Figure 3).

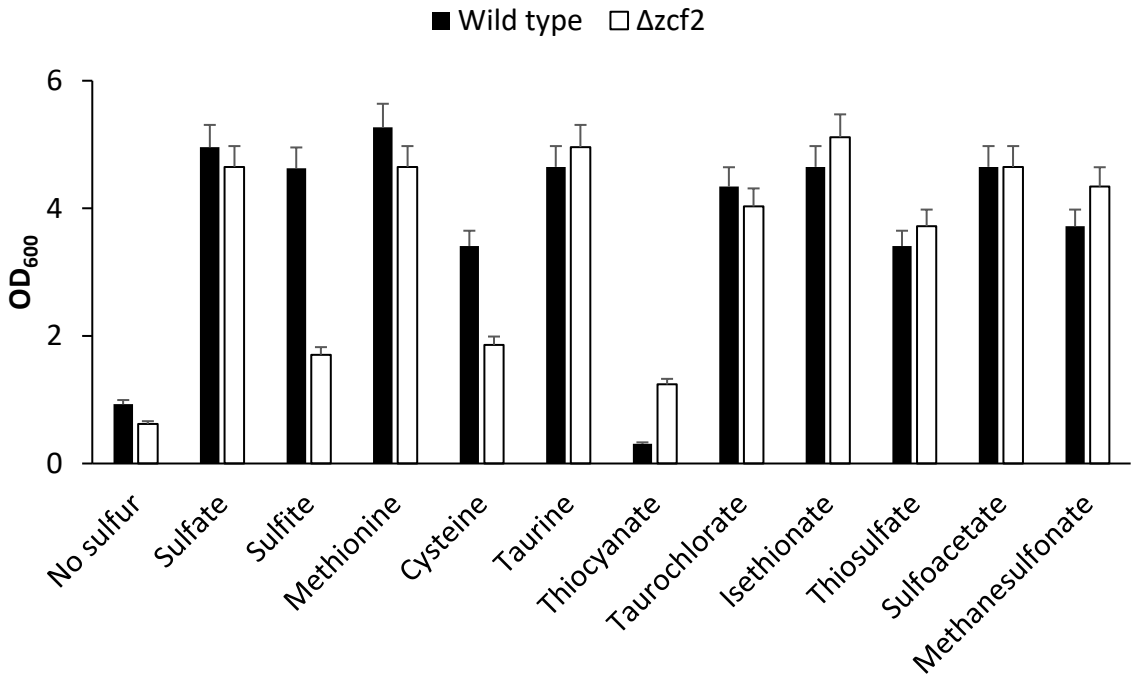

Figure 3-*C. albicans* can grow on a variety of sulfur sources. Overnight cultures of *C. albicans* wild type and *Δzcf2* mutant strains were washed and resuspended in water, and grown at 30°C for 1.5 hours. Following incubation, cells were diluted to an OD_{600} of 0.1 in sulfur-free media supplemented with 2.5 mM of various sulfur sources. Subsequently, the cells were incubated for 21 hours at 30°C and end-point readings of each culture were taken. Results are averages of 3 replicates. Error bars represent standard deviation (SD).

Chapter 4: *Candida albicans* growth is inhibited by sulfite

4.1 *Δzcf2* and *Δssu1* mutants are sensitive to sulfite compared to wild type.

The RSS sulfite has been previously shown to inhibit *C. albicans* under acidic conditions (OGASAWARA et al. 2008). The sulfite transporter, Ssu1, is involved in sulfite resistance in both *S. cerevisiae* and *C. albicans,* as was demonstrated when *Δssu1* mutants showed increased sensitivity to sulfite (AVRAM AND BAKALINSKY 1997; CHIRANAND et al. 2008). Although the transcription factor Cta4 has been shown to regulate the expression of *SSU1* in response to NO, *Δcta4* mutants are not sensitive to sulfite. Therefore, it seemed reasonable that another transcription factor besides Cta4 might regulate *SSU1* expression. To identify this TF, a collection of transcription factor deletion mutants from Homann *et al.* was screened by Rui Zheng for increased sensitivity to sulfite (HOMANN *et al.* 2009). Two transcription factor mutants were found to be sulfite sensitive, *Δzcf2* and *Δleu3.* The latter was sensitive to both nitrite and sulfite, while the *Δzcf2* mutant was not sensitive to nitrite. Work on *LEU3* was suspended due to the inability to reproduce the sulfite sensitive phenotype. To confirm the results of previous lab members as well as establish an assay for analyzing the effects of sulfite on *C. albicans* growth, wild type, *Δzcf2,* and *Δssu1* log phase cultures were grown in the presence and absence of sulfite in a microplate spectrophotometer where optical density readings were taken over a period of several hours. I confirmed that *C. albicans* growth is inhibited by sulfite at pH 4.0, and that *Δzcf2* and *Δssu1* have an increased sensitivity to sulfite compared to wild type (Figure 4A). Thus, Zcf2 is necessary for sulfite resistance.

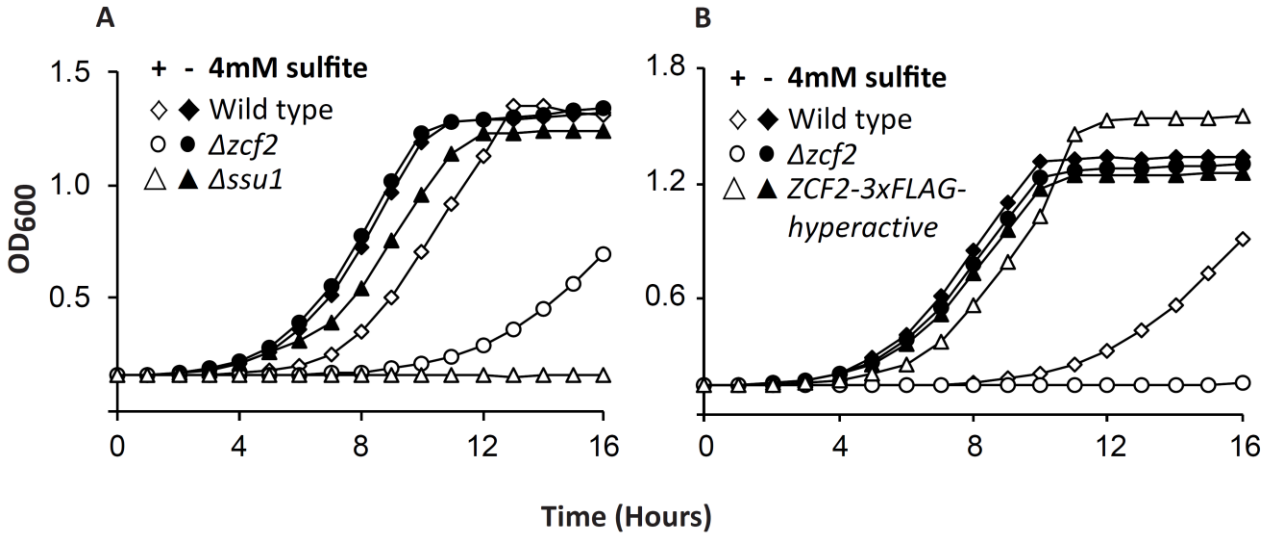

Figure 4- *C. albicans Δzcf2* and *Δssu1* mutants are sensitive to sulfite, and Zcf2 is sufficient for sulfite resistance. Overnight cultures were diluted in YEPD pH4 to an OD_{600} of 0.03, grown to log phase for 3 hours, and then exposed to sulfite concentrations of 0 (filled markers) and 4 mM (empty markers) for 16 hours. Results are averaged from three biological replicates.

4.2 Zcf2 is sufficient for sulfite resistance.

While trying to tag the Zcf2 protein with 3xFLAG for subsequent experiments, I unintentionally created a 'hyperactive' version of Zcf2 that is more resistant to sulfite than wild type (Figure 4B). This tagged *ZCF2* gene was introduced in a *C. albicans* strain that is heterozygous for the *ZCF2* gene and thus the tagged version replaced the remaining copy of *ZCF2*. Sequencing analysis of the *ZCF2-3xFLAG* fusion revealed the following mutations in the *ZCF2* protein coding region: M174G and I256V, where M and I are the wild type amino acids at that position (M: methionine, G: glycine, I: isoleucine, V: valine). These mutations may have caused the resulting protein to regulate genes in a constitutive instead of inducible manner. Regardless of why this tagged Zcf2-3xFLAG protein is 'hyperactive', from this unexpected result, we can conclude that Zcf2 is sufficient for sulfite resistance. Next, I

wanted to look at whether and how *C. albicans* adapts to sulfite, and the role of *Δzcf2* in this process.

Chapter 5: *Candida albicans* can adapt to sulfite following pretreatment with sub-toxic amounts of sulfite, sulfide, or nitrite

5.1 *C. albicans* adapts to sulfite when pretreated with sub-toxic amounts of sulfite.

Adaptation to stress, especially oxidative stress, is seen in a variety of cells where exposure to a subtoxic level of stress induces tolerance to normally toxic levels of stress. *C. albicans* adapts to different stresses, but little is known about whether and how it responds to reactive sulfur species such as sulfite. To test for adaptation to sulfite, *C. albicans* wild type and *Δzcf2* log phase cultures were 'pretreated' with sulfite, that is, cells were grown for 15 minutes in the presence of a subtoxic level of sulfite (0.25 mM). The subtoxic concentration of sulfite was determined by growing *C. albicans* with various amounts of sulfite until the maximum concentration that gives little or no change in growth was found. Following pretreatment, cultures were challenged with higher, toxic levels of sulfite (2, 4, and 8 mM) and growth was measured for 16 hours (Figure 5). Pretreated wild type cultures showed a greater resistance to sulfite than control cultures, while pretreated *Δzcf2* cultures showed the same sensitivity as the untreated control cultures.

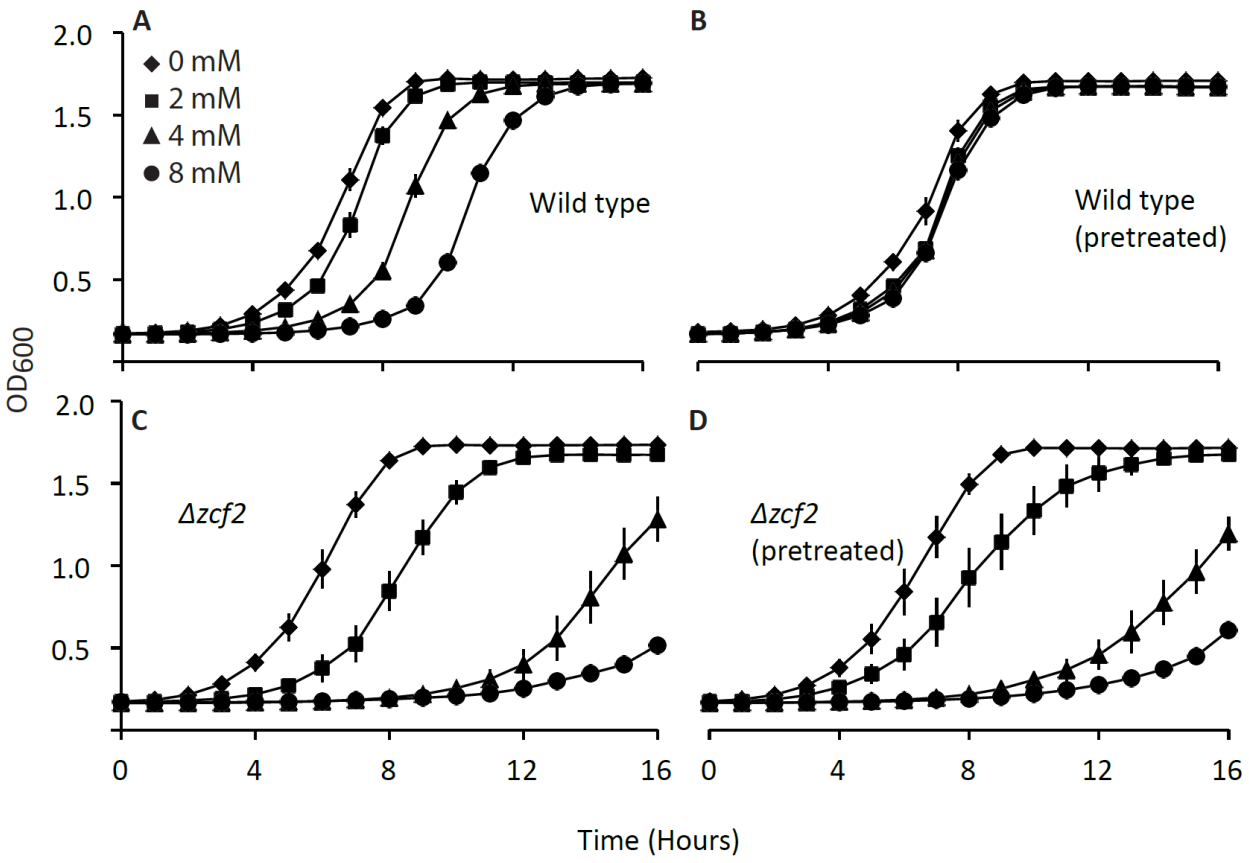

Figure 5- Sulfite-pretreated wild type cells have an increase in sulfite resistance compared to non-pretreated cells. Cultures in log phase were pretreated for 15 minutes with 0.25 mM sulfite at 30°C before being exposed to sulfite concentrations of 0, 2, 4, and 8 mM for 16 hours. Cell density readings were measured at a wavelength of 600nm for (A) non-treated wild type cells, (B) pretreated wild type cells, (C) non-treated *Δzcf2* cells, and (D) pretreated *Δzcf2* cells. Results are averaged from three independent experiments each containing three biological replicates for a total of n=9. Error bars represent standard error of the mean (SEM).

Reinsertion of the *ZCF2* gene in the *Δzcf2* mutant complimented the sulfite adaptation phenotype (Figure 6). Therefore, *C. albicans* adapts to sulfite stress and Zcf2 is necessary for this response. Similarly, pretreated *Δssu1* cultures also showed the same sensitivity to sulfite as control cultures (Figure 7).

The growth curve patterns in Figure 5 are seen often throughout this thesis when showing inhibition by sulfite. That is, in the presence of sulfite, there's a rightward shift in growth compared to growth in the absence of sulfite. Figure 5-A shows this clearly when the curves for 0 mM and 8 mM sulfite are compared. By looking at these curves, it appears that the exponential rate of growth is similar in the presence and absence of sulfite, and that the curves differ regarding when the exponential phase of growth starts, and when saturation is reached. The curve for 0 mM sulfite reaches the beginning of the exponential growth phase sooner than for 8 mM (4 hours versus 8 hours), and reaches saturation sooner (8 hours versus 12 hours). I calculated the growth rate from each curve by finding the slope of the line that best fits each exponential phase. For the 0 mM curve, the slope/growth rate was 0.434 (OD/hour), and for the 8 mM curve the slope/growth rate was 0.543, indicating that sulfite has little effect on growth rate. However, there are a few possible reasons for the difference in time points at which exponential growth is reached in the presence or absence of sulfite. One possibility is that a population of cells is killed by sulfite at the beginning of growth causing the starting number of cells in the presence of sulfite to be lower. Another possibility is that it takes time for cells to produce the proteins needed to grow in the presence of sulfite. Evidence for the latter possibility is provided by the visibly apparent change in slope for the *Δzcf2* mutant in Figure 5-C between growth curves in the absence (0 mM) and presence (4 or 8 mM) of sulfite, which could be due to the expected lower levels of Ssu1 present in this mutant. This can be examined by testing protein levels, such as *SSU1* protein levels, at different time points throughout growth in the presence and absence of sulfite.

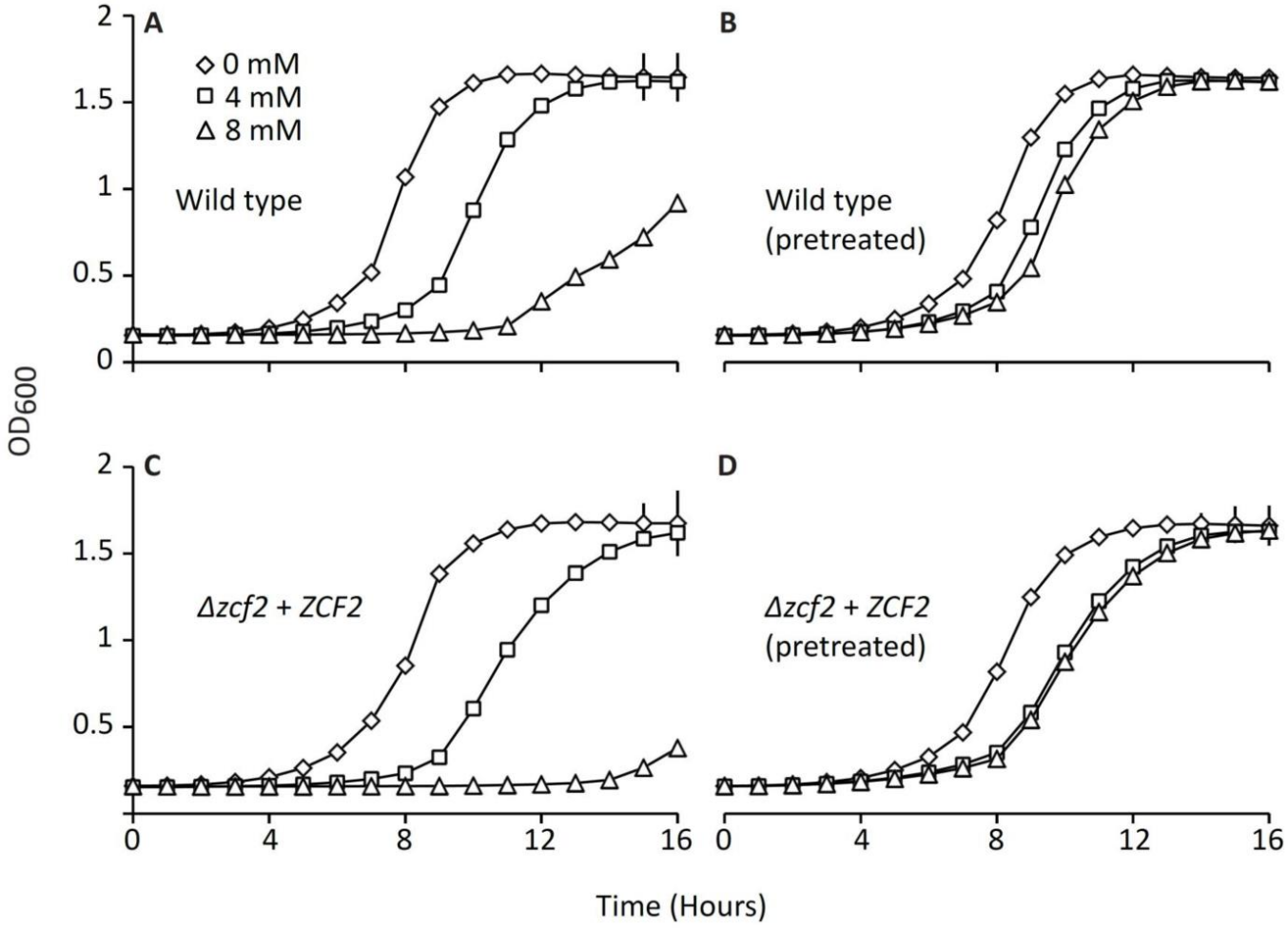

Figure 6- The *Δzcf2* sulfite sensitive phenotype is rescued upon reinsertion of the *ZCF2* gene. Cultures in log phase were pretreated for 15 minutes with 0.25 mM sulfite at 30°C before being exposed to sulfite concentrations of 0, 4, and 8 mM for 16 hours. Cell density readings were measured at a wavelength of 600 nm for (A) non-treated wild type cells, (B) pretreated wild type cells, (C) non-treated *Δzcf2* + *ZCF2* cells, and (D) pretreated *Δzcf2* + *ZCF2* cells. Results are averaged from three biological replicates. Error bars represent SD.

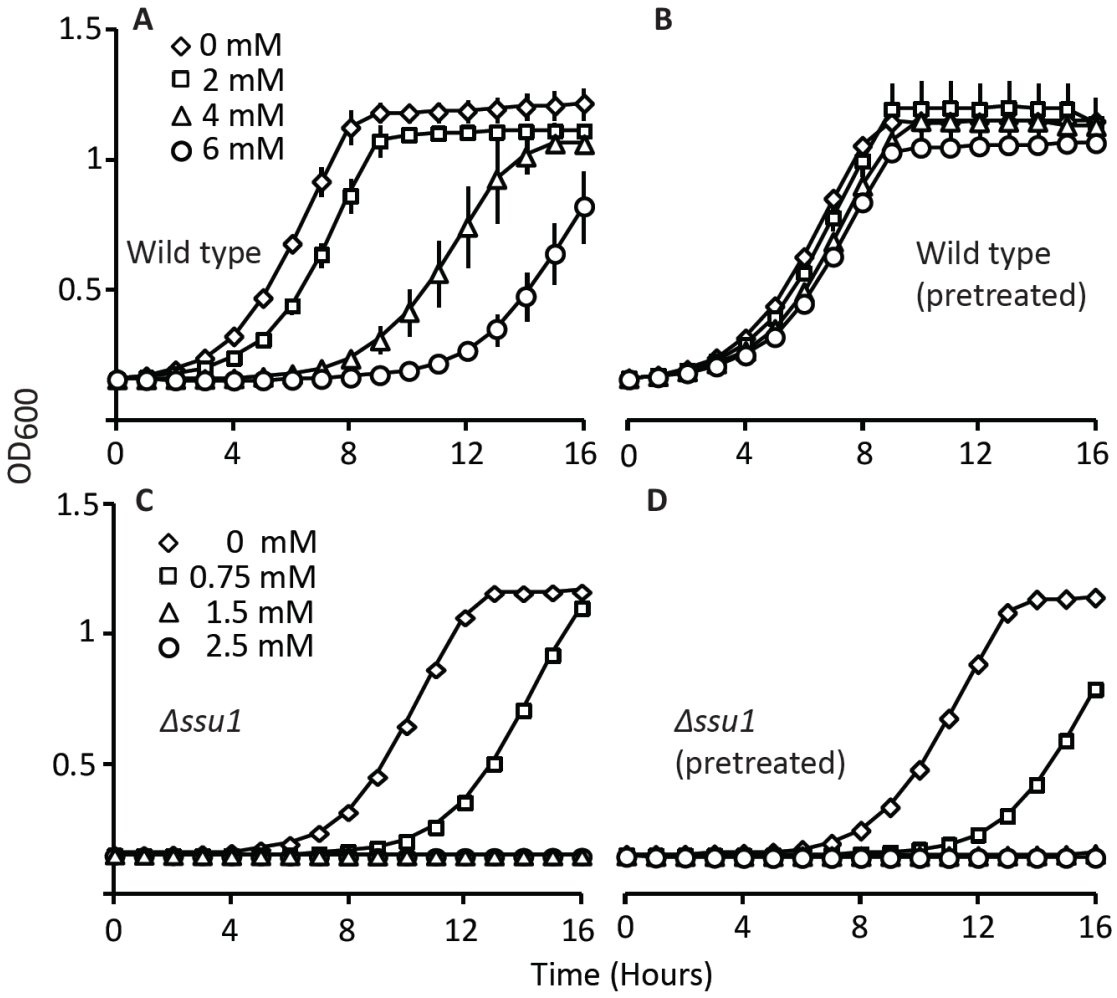

Figure 7- Sulfite pretreated *Δssu1* mutants are not more resistant to sulfite than control cultures. Wild type cultures in log phase were pretreated for 15 minutes with 0.25 mM sulfite at 30°C before being exposed to sulfite concentrations of 0, 2, 4, and 6 mM for 16 hours. *Δssu1* cultures in log phase were pretreated for 15 minutes with 0.25 mM sulfite at 30°C before being exposed to sulfite concentrations of 0, 0.75, 1.5, and 2.5 mM for 16 hours. Cell density readings were measured at a wavelength of 600 nm for (A) non-treated wild type cells, (B) pretreated wild type cells, (C) non-treated *Δssu1* cells, and (D) pretreated *Δssu1* cells. Results are averaged from three biological replicates. Error bars represent SD.

5.2 *Candida albicans* adapts to sulfite when pretreated with sub-toxic amounts of sulfide and nitrite.

Microbes have evolved several ways to protect themselves from oxidative damage involving stress-induced adaptive and cross-protective responses. For example, *S. cerevisiae* exposed to a mild dose of salt acquire tolerance to subsequent severe doses of hydrogen peroxide (GUAN *et al.* 2012). Because *SSU1* is induced in response to a RNS in *C. albicans* (HROMATKA et al. 2005), I examined whether pretreating *C. albicans* with nitrite will induce protection against the RSS sulfite (Figure 8). Cross-protection was observed in nitrite pretreated wild type cells when compared to non-pretreated cells, however, to a much lesser extent than cells pre-treated with sulfite.

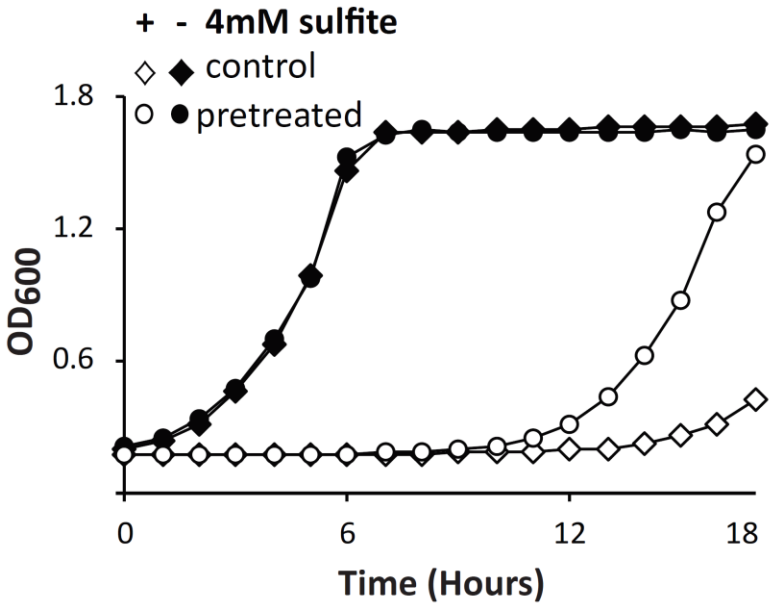

Figure 8- Nitrite pretreated *C. albicans* cells are more resistant to sulfite than control cultures. Wild type cultures in log phase were pretreated for 20 minutes with 0.28 mM nitrite at 30°C before being exposed to sulfite concentrations of 0 (black) and 4 mM (white) for 16 hours. Cell density readings were measured at a wavelength of 600 nm for non-treated (diamond) and pretreated (circle) wild type cells. Results are averaged from three biological replicates.

Sulfide is another RSS that *C. albicans* produced endogenously during sulfate assimilation where sulfite is converted to sulfide. Sulfite conversion to sulfide also accounts for some of the toxicity caused by sulfite, and will be discussed later in this thesis. Because of these observations, I examined whether pretreating *C. albicans* with the sulfide will induce protection against sulfite. Because *ZCF2* plays a role in adaptation to the RSS, sulfite, I also tested its involvement in adaptation when cells are pretreated with another RSS, sulfide. *C. albicans* wild type and *Δzcf2* mutant strains were pretreated with a subtoxic amount of sulfide (0.012 mM) for 15 minutes before challenging with higher sulfite concentrations (Figure 9). The pretreated wild type strain showed a greater resistance to sulfite than control cultures, while pretreated *Δzcf2* mutant cultures showed no change in sulfite resistance. No change in sensitivity to sulfide was observed following sulfide or sulfite pretreatment. Zcf2 is therefore required for the increased sulfite resistance induced by sulfite or sulfide and both RSS induce sulfite resistance.

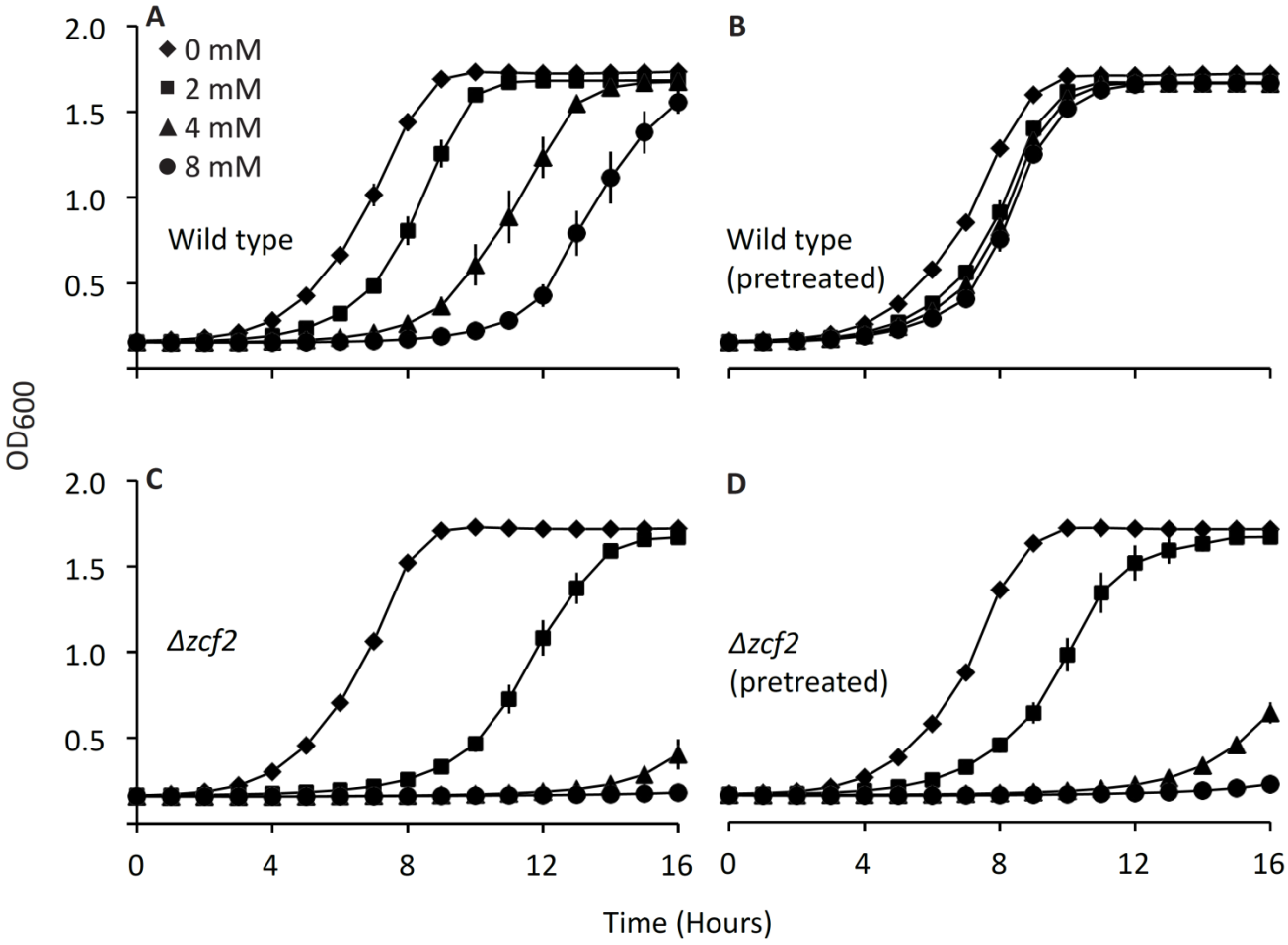

Figure 9- Sulfide-pretreated wild type cells have an increase in sulfite resistance compared to non-pretreated cells. Cultures in log phase were pretreated for 15 minutes with 0.012 mM sulfide at 30°C before being exposed to sulfite concentrations of 0, 2, 4, and 8 mM for 16 hours. Cell density readings were measured at a wavelength of 600 nm for (A) non-treated wild type cells, (B) pretreated wild type cells, (C) non-treated *Δzcf2* cells, and (D) pretreated *Δzcf2* cells. Results are averaged from three independent experiments each containing three biological replicates for a total of n=9. Error bars represent SEM.

5.3 Adaptation Kinetics: How quickly *Candida albicans* can adapt to sulfite and the minimum amount of inducer needed.

After observing that *C. albicans* can adapt to sulfite following sulfite or sulfide pretreatment, the kinetics and pretreatment range of the adaptation were tested, that is

how quickly *C. albicans* can adapt to sulfite and what is the minimum amount of sulfite and sulfide needed. These experiments are useful for predicting whether protein synthesis or activation is occurring during the adaptation process, and whether the non-enzymatic conversion of sulfide to sulfite is playing a role in sulfide-induced adaptation to sulfite. For instance, in the presence of oxygen, sulfide can oxidize to sulfite, thus raising the possibility that sulfide effects are mediated by sulfite (MISHANINA *et al.* 2015). I therefore measured sulfide conversion to sulfite using an enzyme-based assay and determined that, after 30 minutes in water or YEPD at 30°C, 2 mM sulfide yield 4 or 6 μM sulfite, which shows that very little sulfite is generated from sulfide under the experimental conditions used here. By determining the minimum amount of sulfide need to induce adaptation, if the amount is lower than the minimum amount of sulfite needed to induce adaptation, it is likely that sulfide itself is being sensed and causing adaptation and not its conversion to sulfite.

To test the minimum amount of sulfite and sulfide needed to protect against sulfite, cells were pretreated with various concentrations of sulfite ranging from 0 to 0.25 mM, and sulfide ranging from 0 to 0.012 mM. The lowest concentration of sulfite or sulfide that gave a maximal increase in sulfite resistance was 0.031 mM and 0.006 mM, respectively (Figure 10A-B). To test how quickly *C. albicans* adapts to sulfite or what is the minimum pretreatment time needed to pre-adapt cells, wild type strains were pretreated with sulfite or sulfide for various times ranging from 1-20 minutes before being challenged with toxic levels of sulfite. When cells were pretreated with sulfite, complete adaptation was seen after just 7.5 minutes of pretreatment, while cells pretreated with sulfide required a total of 20 minutes (Figure 10C-D) to reach a comparable level of adaptation.

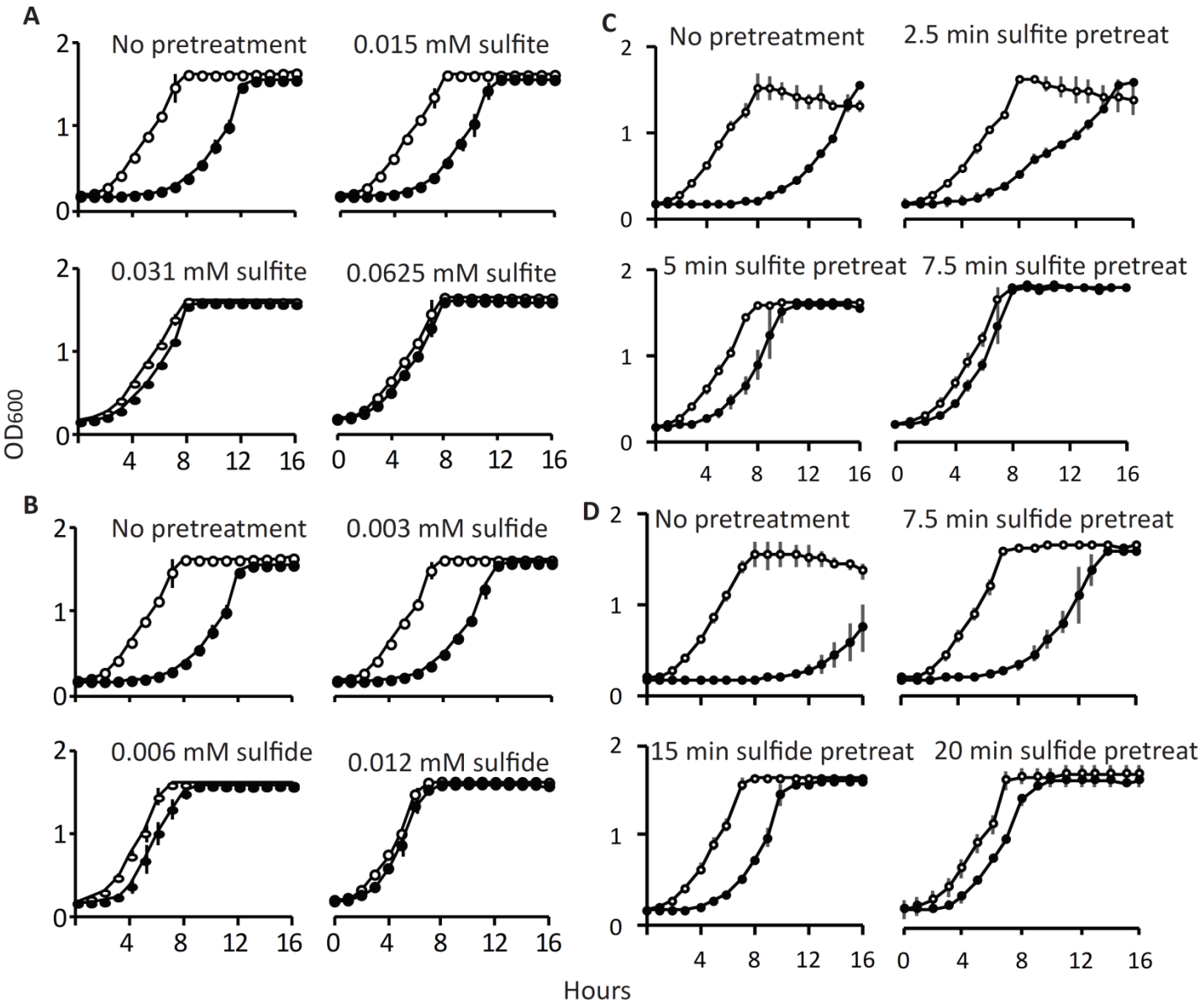

Figure 10- Concentration and time-dependence of sulfite- and sulfide-induced increase in sulfite resistance of *C. albicans*. *C. albicans* grown in the absence (empty markers) and presence (black markers) of 2mM sulfite. (A) *C. albicans* pretreated with 0.015, 0.031, and 0.0625 mM sulfite. (B) *C. albicans* pretreated with 0.003, 0.006, and 0.012 mM sulfide. (C) *C. albicans* pretreated for 0, 2.5, 5, and 7.5 minutes with 0.25 mM sulfite. (D) *C. albicans* pretreated for 0, 7.5, 15 and 20 minutes with 0.012 mM sulfide. Results are averages of 3 biological replicates. Errors bars represent SD.

Therefore, even if the entire 0.006 mM sulfide was converted to sulfite, that amount of sulfite is not enough to induce sulfite resistance. These results argue that non-enzymatic conversion of sulfide to sulfite does not play a role in the *ZCF2*-dependent induction of sulfite resistance, and that *C. albicans* senses sulfide. However, the longer time course of sulfide-induced sulfite adaptation suggests that sulfide has to be converted to another chemical to effect this response.

Chapter 6: Sulfite induces a specific set of genes in *C. albicans*

6.1 Genes induced and repressed in response to sulfite.

The genes and pathways in *C. albicans* involved in resistance to ROS and RNS have been previously studied through transcriptional response analyses (HROMATKA *et al.* 2005; ENJALBERT *et al.* 2006). However, little is known about the genes that are induced or repressed in response to sulfite. Genome-wide transcriptional profiling in *S. cerevisiae* following exposure to sulfite showed the upregulation of *SSU1* and *PDC1* (PARK AND HWANG 2008). *PDC1* encodes for pyruvate decarboxylase, Pdc1, which catalyzes the reduction of pyruvate into acetaldehyde. Acetaldehyde reacts with sulfite to form the non-toxic compound acetaldehyde hydroxysulfonate (XU *et al.* 1994). Therefore, it is predicted that *S. cerevisiae* adapts to sulfite stress by increasing sulfite efflux from cells and detoxifying sulfite by its reactions with a chemical intermediate in ethanol fermentation.

Similarly, to determine the genes induced by sulfite in *C. albicans,* and which genes require Zcf2 for sulfite-induction, I performed an Agilent DNA microarray-based analysis of mRNA extracted from wild type and *Δzcf2* cells exposed to sulfite. *C. albicans* cells were cultured for 15 minutes in the presence or absence of 0.25 mM sulfite, a subtoxic

concentration, and the ratio of different mRNAs +/- sulfite analyzed by hybridization to *C. albicans* DNA microarrays. In the wild type strain, about 1000 genes showed a statistically significant difference of at least 2-fold change in level of mRNA between the control and sulfite-treated cultures. Of those genes, 492 genes increased in expression and 511 genes decreased. The 32 genes whose expression changed by at least 8-fold in response to subtoxic sulfite include those predicted to be involved in alternative oxidation (*AOX2*), sulfite removal and detoxification (*SSU1*), nitrogen/ammonia metabolism and oligopeptide transport (*AMO1, GAP2, CAN2, IFC3,* and *IFC1*), and ribosome biogenesis (orf19.1862) (Table 1). 25 of 30 of the genes showing an 8-fold or greater reduction in expression are predicted to have a role in ribosome or tRNA biogenesis (Table 2). Therefore, the transcriptional response to sulfite in *C. albicans* differs greatly from that reported for *S. cerevisiae* (PARK AND HWANG 2008).

TABLE 1- Selected sulfite induced genes involved in alternative respiration, sulfite removal, nitrogen catabolite repression, and ribosome biogenesis in wild type *C. albicans*.

Gene group and orf no. (Name)	Proposed gene function	Fold Induction
Alternative respiration		
orf19.4773 (*AOX2*)	Alternative oxidase	194
Sulfite removal		
orf19.7313 (*SSU1*)	Sulfite transporter	28
orf19.1867 (*SSU12)*	Sulfite transporter	13
Nitrogen Metabolism		
orf19.5784 (*AMO1*)	Peroxisomal copper amine oxidase	26
orf19.6993 (*GAP2*)	Amino acid and polyamine permease	17
orf19.111 (*CAN2*)	Arginine transporter	16
orf19.3749 (*IFC3*)	Peptide transporter	14
orf19.3746 (*OPT2)*	Peptide transporter	14
orf19.313 (*DAL4*)	Allantoin permease	14
orf19.360 (*FUR4*)	Uracil permease	12
Ribosome biogenesis		
orf19.1862 (*RTC3*)	Ribosome metabolism	26

Selected genes significantly differentially expressed in sulfite-treated wild type *C. albicans* cells. (t-test: n=8)

Table 2-A Genes involved in ribosome biogenesis are down-regulated after exposure to sulfite. List of 26 of 30 genes down-regulated by more than 8-fold in wild type.

C. albicans ORF19 No.	*C. albicans* name	*S. cerevisiae* homolog	Category	Fold Repression	
				Wild type	*Δzcf2*
orf19.1691		*RCF2*	cytochrome oxidase assembly	-16.5	-6.2
orf19.5020			TF distantly related to ScSKO1	-13.9	-18.5
orf19.5507	*ENP1*	*ENP1*	Ribosome biogenesis	-13.5	-11.4
orf19.4342		*SUT1*	TF for sterol uptake	-12.6	-3.8
orf19.5010	*DIM1*	*DIM1*	Ribosome biogenesis	-12.4	-10.7
orf19.1091	*NOP8*	*NOP8*	Ribosome biogenesis	-11.9	-16.6
orf19.2830	*RRP9*	*RRP9*	Ribosome biogenesis	-11.7	-14.8
orf19.6686	*ENP2*	*ENP2*	Ribosome biogenesis	-11	-10.6
orf19.661	*KRR1*	*KRR1*	Ribosome biogenesis	-10.9	-10.5
orf19.3159	*UTP20*	*UTP20*	Ribosome biogenesis	-10.9	-9.8
orf19.5500	*MAK16*	*MAK16*	Ribosome biogenesis	-10.8	-17.2
orf19.7197		*NOC3*	Ribosome biogenesis	-9.9	-8.5
orf19.5207		*DPH1*	Ribosome biogenesis	-9.9	-12.8
orf19.2167		*RRP14*	Ribosome biogenesis	-9.7	-12.1
orf19.6298	*SPB4*	*SPB4*	Ribosome biogenesis	-9.6	-11.8
orf19.2654	*RMS1*	*RKM4*	Ribosome biogenesis	-9.5	-8.3
orf19.59	*REI1*	*REI1*	Ribosome biogenesis	-9.3	-10.8
orf19.1566	*UTP21*	*UTP21*	Ribosome biogenesis	-8.9	-6.5
orf19.2385	*KTI12*	*KTI12*	Pol II Elongator complex, tRNA modification	-8.8	-6.4
orf19.5959	*NOP14*	*NOP14*	Ribosome biogenesis	-8.8	-10.4
orf19.6886		*NOP53*	Ribosome biogenesis	-8.7	-8.6
orf19.1923	*RRN3*	*RRN3*	Ribosome biogenesis	-8.5	-4.2
orf19.7154	*UTP18*	*UTP18*	Ribosome biogenesis	-8.5	-6.3
orf19.3088		*ATC1*	bZIP Transcription factor	-8.4	-5.7
orf19.603	*IMP4*	*IMP4*	Ribosome biogenesis	-8.3	-8.2
orf19.4760		*HPM1*	Ribosome biogenesis	-8.3	-9
orf19.7650	*LTV1*	*LTV1*	Ribosome biogenesis	-8.2	-6.1
orf19.2185	*NSA1*	*NSA1*	Ribosome biogenesis	-8.2	-6.1
orf19.563	*RRP1*	*RRP1*	Ribosome biogenesis	-8.1	-9.1
orf19.1388		*NOP16*	Ribosome biogenesis	-8	-8

Table 2-B Function of genes down-regulated after exposure to sulfite. Function of 26 of 30 genes down-regulated by more than 8-fold in wild type.

C. albicans ORF19 No.	Function
orf19.1691	cytochrome oxidase assembly
orf19.5020	TF distantly related to ScSKO1
orf19.5507	Protein required for pre-rRNA processing and 40S ribosomal subunit synthesis
orf19.4342	TF for sterol uptake
orf19.5010	Putative 18S rRNA dimethylase; predicted role in rRNA modification and processing
orf19.1091	Role in ribosomal large subunit biogenesis
orf19.2830	Ribosomal protein
orf19.6686	Putative nucleolar protein
orf19.661	Putative nucleolar protein
orf19.3159	Putative snoRNA-binding protein
orf19.5500	Putative constituent of 66S pre-ribosomal particles
orf19.7197	Putative intranuclear transport and DNA replication mediator
orf19.5207	Diphthamide biosynthesis
orf19.2167	Ribosomal small subunit biogenesis and cytosolic large ribosomal subunit
orf19.6298	Putative ATP-dependent RNA helicase
orf19.2654	Putative lysine methyltransferase
orf19.59	Putative cytoplasmic pre-60S factor
orf19.1566	Putative U3 snoRNP protein
orf19.2385	Pol II Elongator complex, tRNA modification
orf19.5959	Putative nucleolar protein
orf19.6886	Role in maturation of LSU-rRNA from tricistronic rRNA transcript, ribosomal large subunit export from nucleus and nucleolus localization
orf19.1923	Pol I binding to rRNA gene
orf19.7154	Putative U3 snoRNA-associated protein
orf19.3088	bZIP Transcription factor
orf19.603	Putative small subunit processome component
orf19.4760	Methyltransferase involved in a novel 3-methylhistidine modification of ribosomal protein Rpl3p
orf19.7650	GSE (GAP1 Sorting in the Endosomes) complex, ribosomal small subunit export from nucleus
orf19.2185	Putative 66S pre-ribosomal particles component
orf19.563	Putative nucleolar protein; constituent of pre-60S ribosomal particles
orf19.1388	Putative 66S pre-ribosomal particle component

6.2 Genes induced in response to sulfite that show Zcf2-dependence.

Of the 492 most induced genes in response to sulfite, 84 of them showed at least a 2-fold increase (19) or decrease (65) in the *Δzcf2* strain relative to wild type. The 65 genes that show Zcf2-dependent sulfite induction fit into categories like those observed to be induced in wild type in response to sulfite: sulfite reduction or removal, alternative oxidation, and nitrogen/ammonia metabolism (Table 3). Thus, the sulfite removal genes and most of the ammonia metabolism genes are Zcf2-dependent.

TABLE 3- In response to sulfite, Zcf2-dependent genes fit into 3 categories: Sulfite removal or reduction, ammonium metabolism, and mitochondrial respiration. 28 of the 65 Zcf2-dependent genes that were down-regulated more than 2-fold in the Δ*zcf2* mutant versus wild type. (t-test: n=8)

Gene group and orf no. (Name)	Proposed gene function	Fold induction in		Ratio of fold induction in wild type vs. Δ*zcf2*
		Wild type	Δ*zcf2*	
Sulfite removal or reduction				
orf19.5811 (*MET1*)	Uroporphyrin-3 C-methyltransferase (siroheme)	15.6	1.4	11.1
orf19.7313 (*SSU1*)	Sulfite transporter	28.0	2.5	11.2
orf19.4076 (*MET10*)	Sulfite reductase	4.9	0.5	9.8
orf19.4099 (*ECM17*)	Sulfite reductase β-subunit	3.7	0.9	4.1
Ammonium metabolism				
orf19.6993 (*GAP2*)	Amino acid and polyamine permease	17.6	0.9	19.6
orf19.5784 (*AMO1*)	Peroxisomal copper amine oxidase	25.4	1.7	14.9
orf19.4063 (*GPT1*)	GABA/polyamine transporter	8.6	0.7	12.3
orf19.5672 (*MEP2*)	Ammonium permease	10.2	1.0	10.2
orf19.780 (*DUR1,2*)	Urea amidolyase	12.1	1.8	6.7
orf19.6570 *(NUP*)	Nucleoside permease	3.8	0.6	6.3
orf19.313 (*DAL4*)	Allantoin permease	14.0	2.7	5.2
orf19.781 (*DUR3*)	Spermidine transporter	4.5	1.0	4.5
orf19.2882 (*XUT1)*	Xanthine-uric acid transporter	2.2	0.5	4.4
orf19.111 (*CAN2*)	Arginine transporter	16.1	3.7	4.3
orf19.360 (*FUR4*)	Uracil permease	12.4	3.0	4.1
orf19.4716 (*GDH3*)	Glutamate dehydrogenase	3.0	1.0	3.0
orf19.97 (*CAN1*)	Amino acid permease	6.3	2.2	2.9
orf19.2474 (*PRC3*)	Carboxypeptidase	3.2	1.2	2.7
orf19.1275 (*GAT1*)	Transcription factor (regulator of nitrogen utilization)	10.1	4.3	2.3
orf19.6950	Arginine exporter from vacuole	3.2	1.5	2.1
Mitochondrial respiration				
orf19.1114	Cytochrome c oxidase subunit	2.0	0.6	3.3
orf19.4841 (*SHY1*)	Cytochrome c oxidase biosynthesis	3.5	1.3	2.7
orf19.1416 (*COX11*)	Cytochrome oxidase assembly	3.0	1.1	2.7
orf19.1667.1	Cytochrome oxidase assembly	2.9	1.2	2.4
orf19.409	Cytochrome c oxidase subunit	4.8	2.0	2.4
orf19.4773 (*AOX2*)	Alternative oxidase	198.5	84.1	2.4
orf19.1336.2	Mitochondrial respiratory chain complex assembly	2.3	1.0	2.3
orf19.4774 (*AOX1*)	Alternative oxidase	10.8	5.2	2.1

6.3 Deletion of genes most induced by sulfite (*AOX2, AMO1,* and orf19.3120) does not result in a sulfite-sensitive phenotype.

Of the most upregulated genes in response to sulfite, only *Δssu1* showed a sulfite sensitive phenotype. When three other strongly upregulated genes: *AOX2, AMO1,* and orf19.3120, were deleted from *C. albicans,* no sulfite sensitive-phenotype was observed. Likewise, when orf19.3120 was deleted from an *Δssu1* mutant, no synergistic sensitivity to sulfite was seen. Therefore, the role in sulfite detoxification of some of the most upregulated genes remains unclear, although the induction of *AOX2* indicates that sulfite is potentially affecting mitochondrial respiration.

6.4 Zcf2-dependent induction of nitrogen metabolism genes in response to sulfite does not indicate a role for Zcf2 in nitrogen starvation or ammonia release.

The induction of nitrogen metabolism genes indicates that the cells are responding similarly to sulfite as they would to nitrogen starvation, even though the cells were grown in nitrogen-rich medium. Additionally, the Zcf2-dependent induction of nitrogen metabolism genes in response to sulfite raised the possibility that Zcf2 plays a role in nitrogen metabolism. To begin testing this, I grew wild type and *Δzcf2* mutant strains 'nitrogen-limited' conditions in media containing various amounts of ammonia (0 to 10 mM). However, no difference between wild type and mutant strains was observed. Wild type and *Δzcf2* mutant strains were also grown on different nitrogen sources (glutamic acid, ammonia acetate, and urea), and again, *Δzcf2* showed no phenotype. Lastly, I measured whether *Δzcf2* would have a defect in releasing ammonia. Because pH adaptation is critical for virulence in distinct host niches, *C. albicans* actively releases ammonia to neutralize the environment from either acidic or alkaline pHs (VYLKOVA *et al.* 2011).

However, when I analyzed ammonia production in *C. albicans* wild type and *Δzcf2* mutant cells, no difference was observed (Figure 11).

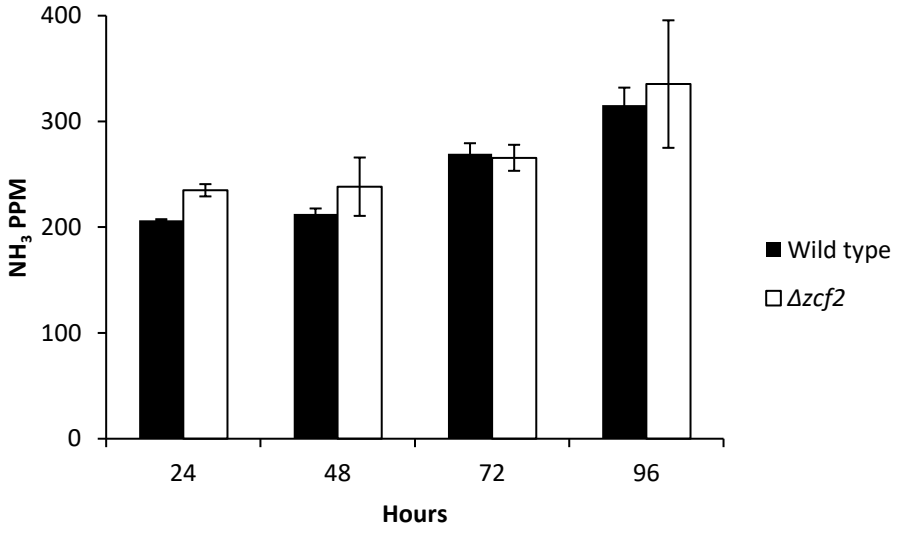

Figure 11- Ammonia release by *C. albicans* wild type and *Δzcf2* strains. *C. albicans* wild type-*Arg*$^{+}$ and *Δzcf2-Arg*$^{+}$ strains were spotted on 'GM-BCP' agar plates containing the pH indicator bromocresol purple as well as sulfite. Subsequently, the spots were left to dry and reservoirs containing 10% citric acid were placed underneath the colonies. Cells were incubated at 37°C and 20 µl samples from the acid traps were collected at 24, 48, and 72 hours, and stored at -20 °C. Parts per million (ppm) concentrations of ammonia were calculated based on a standard curve. Results are averages of 3 biological replicates. Error bars represent SD.

Chapter 7: Sulfite conversion to sulfide mediates the toxicity of sulfite in *Candida albicans*

Induction of the alternative oxidase gene *AOX2,* suggests inhibition of cytochrome c oxidase, in this case by sulfite (XIAO *et al.* 2010; NICHOLLS *et al.* 2013). *AOX2* encodes alternative oxidase, which provides a distinct oxidase pathway from cytochrome c oxidase

located in the mitochondrial electron transport chain. In the sulfate assimilation pathway (Figure 2), sulfite is converted to sulfide by the Ecm17/Met10 sulfite reductase (THOMAS AND SURDIN-KERJAN 1997; LI *et al.* 2013). Sulfide is a known inhibitor of cytochrome c oxidase and is toxic to *C. albicans;* the *Δzcf2* mutant is more sensitive to sulfide (Figure 12). An *Δecm17* mutant is highly resistant to sulfite, suggesting that sulfite conversion to sulfide mediates the toxicity of sulfite (Figure 13).

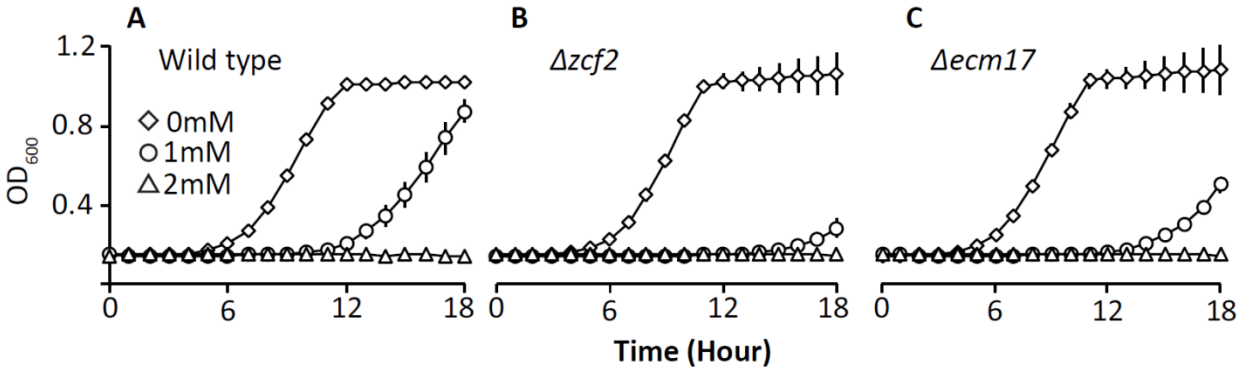

Figure 12- Sulfide is toxic to *C. albicans* strains. Overnight cultures (**(A)** wild type, **(B)** *Δzcf2,* and **(C)** *Δecm17*) were diluted in YEPD pH4 to and OD_{i600} of 0.03, grown to log phase for 3 hours, and then exposed to 0, 1, and 2 mM sulfide for 18 hours. Results are averages of 3 biological replicates. Error bars represent SD.

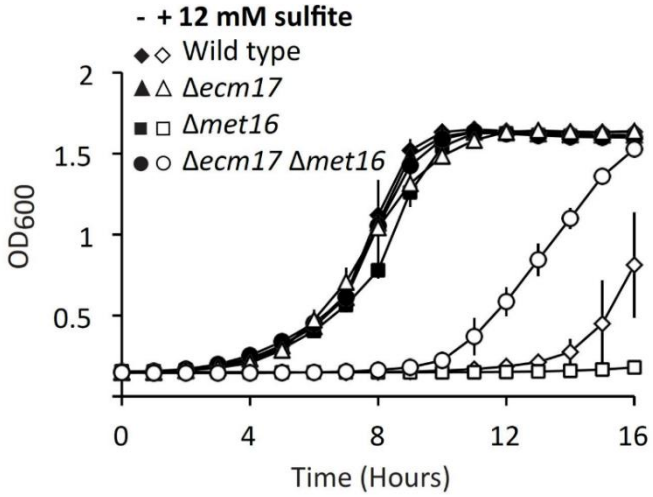

Figure 13- *C. albicans* lacking *ECM17* are resistant to sulfite. Cell cultures in log phase were challenged for 16 hours with 0 mM and 12 mM sulfite (filled and empty markers, respectively). Results are averages of 3 biological replicates (n=3). Error bars represent SD.

The *Δecm17* sulfite-resistant phenotype is consistent with the idea that sulfite conversion to sulfide mediates sulfite toxicity, however, there is another possible explanation. If there is flux of metabolites through the sulfate assimilation pathway, the block at the sulfite reductase step in the *Δecm17* mutant strain is predicted to cause accumulation of sulfite to an extent that would induce sulfite adaptation. To test this possibility, I constructed a strain with homozygous deletions of *MET16* and a double-deletion strain of both *ECM17* and *MET16.* The natural sulfite accumulation in an *Δecm17* mutant strain is predicted to be reduced when *MET16* is also deleted. As predicted, the *Δecm17* and *Δecm17 Δmet16* strains fail to produce sulfide from sulfite, as assessed with a bismuth sulfide-based detection method, and *Δmet16*, *Δecm17* and *Δecm17 Δmet16* also had the expected methionine auxotrophy phenotype (Figure 14).

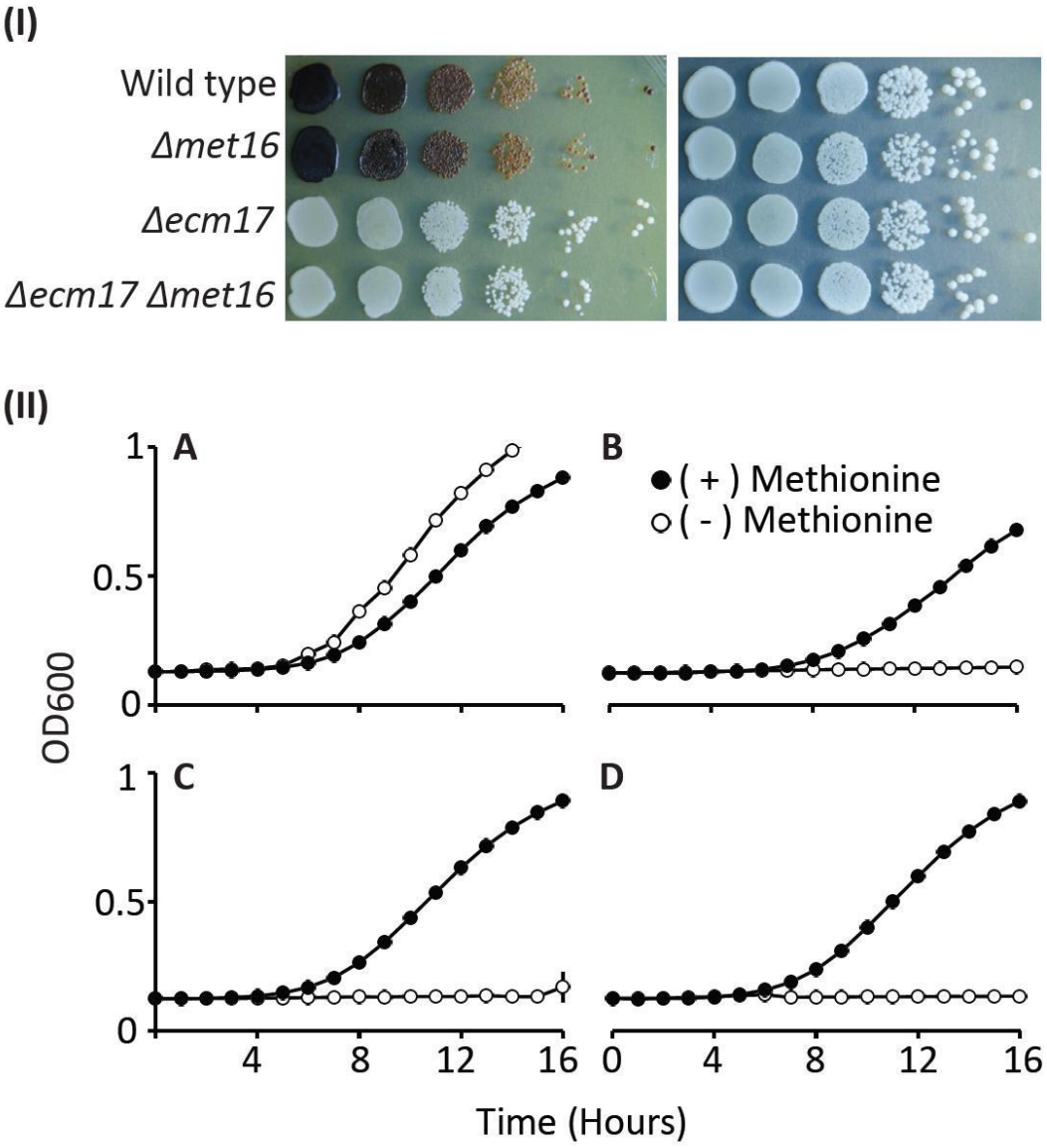

Figure 14 - *C. albicans Δecm17* and *Δecm17 Δmet16* mutants do not produce sulfide and are methionine auxotrophs. (I) Overnight cultures of wild type, *Δmet16*, *Δecm17*, and *Δecm17 Δmet16* were serially diluted and spotted on BiBAGY agar (left) containing bismuth and sodium sulfite. Black pigmented colonies indicate the formation of sulfide. Cultures were also plated on YEPD media (right) for control. **(II) .** Overnight cultures **(A)** wild type, **(B)** *Δmet16,* **(C)** *Δecm17,* and **(D)** *Δecm17 Δmet16*) were washed, incubated in water for 45 minutes, and then resuspended in SD medium with or without 0.75 mg/ml methionine. Results are averages of 3 biological replicates.

The *Δmet16* strain displayed wild type sulfite sensitivity while the double deletion *Δecm17 Δmet16* strain showed a phenotype that was intermediate between the wild type and the *Δecm17* mutant strain (Figure 13). These results indicate that both sulfide and sulfite accumulation play a role in sulfite-induced toxicity. This is because even though natural sulfite accumulation was reduced in the *Δecm17 Δmet16* strain, exogenous sulfite was still not as toxic as it would be in a sulfide-forming strain. Sulfite is also toxic to *S. cerevisiae*, however, deletion of the *ECM17* ortholog, *MET5,* had no effect on the sulfite sensitivity of this yeast, further indicating that *C. albicans* and *S. cerevisiae* tolerate sulfite in mechanistically distinct ways (Figure 15).

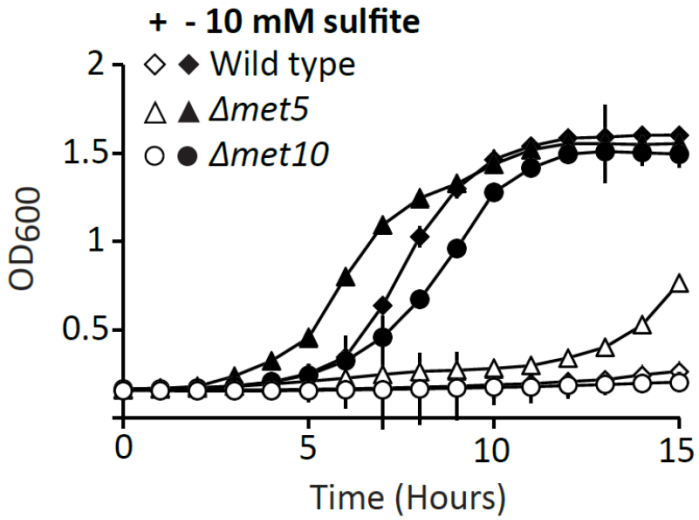

Figure 15- *S. cerevisiae Δmet5* and *Δmet10* mutants show wild type sensitivity to sulfite. Overnight cultures of wild type, *Δmet5,* and *Δmet10 S. cerevisiae* were diluted in YEPD pH4 to an OD_{i600} of 0.03, grown to log phase for 3 hours, and exposed to 0 (black markers) and 10 mM (empty markers) sulfite for 15 hours. Results are averages of 3 biological replicates. Error bars represent SD.

Chapter 8: Zcf2-dependent expression of *SSU1* reporter is induced by several reactive sulfur species.

The finding that sulfite or sulfide pretreatment resulted in sulfite adaptation raised questions about which molecule Zcf2 senses. Therefore, a luciferase-based reporter system was used where 1 kilobase of the *SSU1*-promoter region was placed upstream of a modified version of a *Gaussia princeps* luciferase gene, *gLUC* (ENJALBERT et al. 2009). The luciferase reporter is fused to *C. albicans PGA59,* which encodes a cell wall protein. Luciferase expressed from this fusion localizes to the cell surface, allowing for detection of luciferase luminescence from extracellular coelenterazine by intact cells whenever *SSU1* is induced. *SSU1-gLUC* was transformed into various *C. albicans* mutant strains, some of which were deficient in sulfite and sulfide production, to assess which exogenously added RSS caused Zcf2-dependent expression of *SSU1.*

Wild type showed increased *SSU1-gLUC* induction in response to subtoxic levels of sulfite (1 mM) or sulfide (0.75 mM) compared to control cultures, while the *Δzcf2* strain showed no induction by either RSS (Figure 16A). These results indicate that Zcf2 is involved in sensing both these signals, however, it is possible that sulfite induction of *SSU1-gLUC* expression was due to the reduction of sulfite to sulfide by sulfite reductase, Ecm17. To test this, *SSU1-gLUC* induction was tested in a *Δecm17 Δmet16* strain, which is incapable of producing endogenous sulfite and sulfide. *SSU1-gLUC* expression increased in the *Δecm17 Δmet16* mutant in response to added sulfite or sulfide, suggesting that both RSS act as signals. However, induction by sulfite is lower in the *Δecm17 Δmet16* than in wild type, indicating that part of sulfite induction of *SSU1* is due to the conversion of sulfite to sulfide. In addition to sulfite and sulfide, cysteine has been shown to induce Zcf2-dependent *SSU1*

expression in *C. albicans* (HENNICKE et al. 2013). To confirm this finding, *SSU1-gLUC* induction by cysteine (1.25 mM) in SD medium was examined in wild type, *Δzcf2,* and *Δcdg1* strains. *C. albicans* strains lacking *CDG1,* cysteine dioxygenase, cannot convert cysteine to sulfite (HENNICKE *et al.* 2013). *SSU1-gLUC* induction by cysteine was observed in all three strains at varying levels (Figure 16B). The *Δzcf2,* and *Δcdg1* strains showed significantly reduced induction compared to wild type, confirming previous work showing that *SSU1* regulation by cysteine is partially dependent on Zcf2 as well as the Cdg1-dependent conversion of cysteine to sulfite (HENNICKE *et al.* 2013).

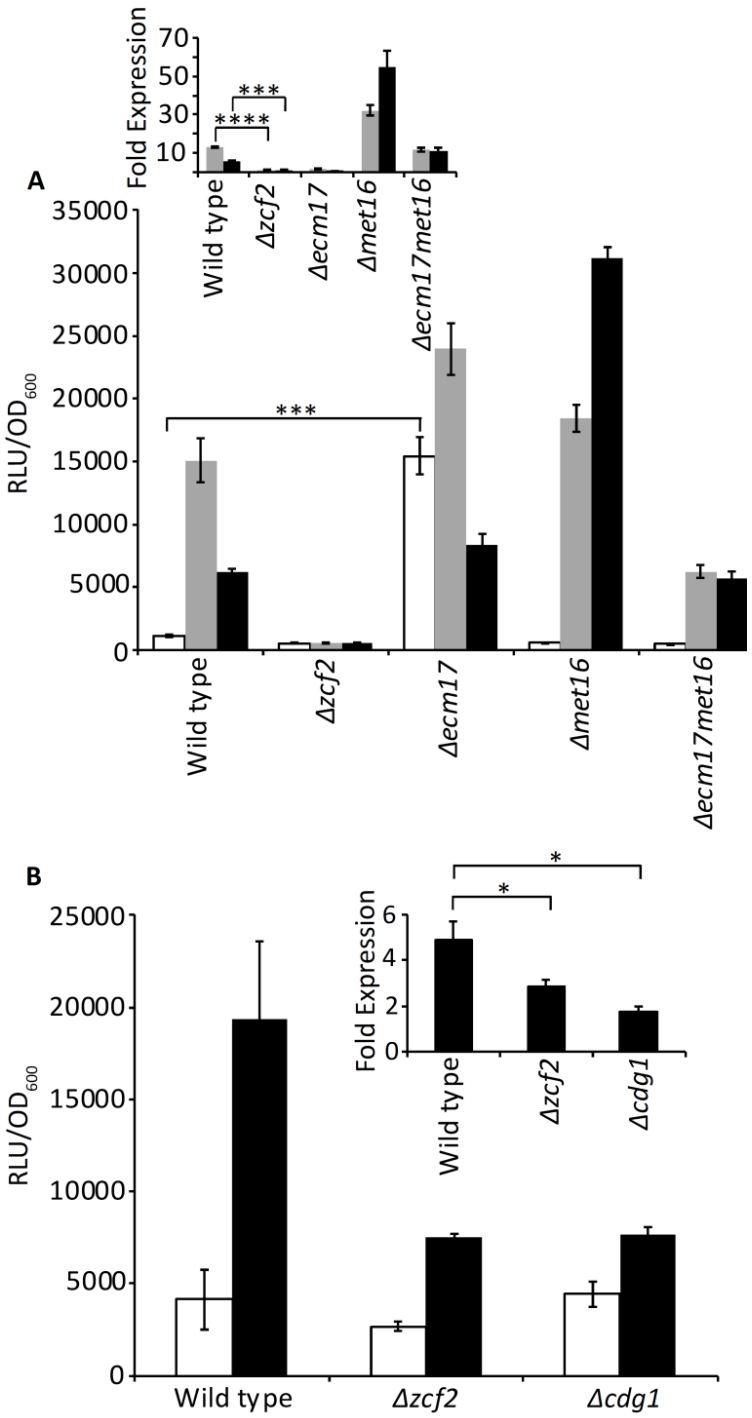

Figure 16- *SSU1-gLUC* induction by sulfite and sulfide is Zcf2-dependent. Cultures in log phase were grown in the presence or absence (white) of **(A)** 1.0 mM sulfite (grey) or 0.5 mM sulfide (black) for 30 minutes, or **(B)** 1.25 mM cysteine (black) for 1 hour, at 30°C. *SSU1-gLUC* induction, shown in Relative Luminescence Units (RLU), by sulfite and sulfide was observed in the wild type strain, as well as in cells unable to endogenously produce

sulfite (*Δmet16*), sulfide (*Δecm17*), or either sulfite and sulfide (*Δecm17 Δmet16*). No *SSU1-gLUC* induction was observed in the *Δzcf2* mutant. Induction by cysteine was seen in all three strains tested. The insets shown in **(A)** and **(B)** show fold expression change using the ratio: $((RLU/OD_{600inducer})/(RLU/OD_{600control}))$. Results are averages of three biological replicates. (*), (***) and (****) asterisks indicate that the detected differences were significant: (P<0.01), (P<0.001) and (P<0.0001) respectively.

Moreover, as an additional control, *ACT1-gLUC* expression was examined in response to sulfite or sulfide; only a small decrease in *ACT1-gLUC* expression was observed (Figure 17).

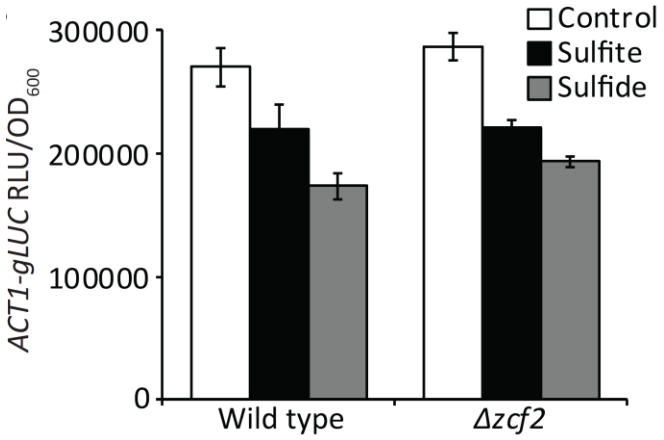

Figure 17-*ACT1-gLUC* induction is reduced by sulfite and sulfide. Log phase cultures of wild type and *Δzcf2* cells containing the *ACT1-gLUC* reporter were grown in the presence or absence (white) of 1.0 mM sulfite (black) or 0.5 mM sulfide (gray) for 30 minutes, at 30°C. Results are averages of two biological replicates, and the experiment has been repeated several times.

Chapter 9: Sulfite induction of *SSU1* is independent of nitrite induction

9.1 Motif mutations in the *SSU1-gLUC* reporter affect sulfite and nitrite induction separately.

In *S. cerevisiae,* the transcription factor Fzf1 mediates responses to both RNS and RSS, however, it is not known if *C. albicans* also uses a common pathway to respond to these molecules. Sulfite and nitrite induction of *SSU1* via the transcription factors Cta4 and Zcf2, respectively, suggests that Zcf2 regulates *SSU1* through different binding sites. Locating these binding sites will also determine if *SSU1* expression is regulated by nitrite, sulfite, and cysteine through common or parallel pathways. In *C. albicans,* the regulatory region of a gene is typically within 1 kilobase upstream of the start codon of the ORF. Therefore, mutations were introduced in the *SSU1* promoter region of the *SSU1-gLUC* reporter and the effects of the mutations were examined in response to reactive chemicals known to activate *SSU1* expression such as sulfite, sulfide, cysteine, or nitrite.

Six upstream sequence motifs, or putative transcription factor binding sites were tested. Three of the motifs (motifs 1-3) were found via the motif-detecting program, MEME, using data from the Zcf2-dependent sulfite regulon revealed from the microarray experiment (Bailey and Elkan 1994). The other three motifs (motifs 4-6) were determined using existing information on binding motifs for other zinc cluster family members: motif 4 consisted of a pair of palindromic CGG nucleotide triplets separated by 14 nucleotides ($CGGN_{14}CCG$); this is similar to a previously identified common binding element ($CGGN_xCCG$) found for the class of zinc cluster transcription family that includes the well-studied Gal4 transcription factor, and Zcf2 (Giniger *et al.* 1985; Liang *et al.* 1996;

MacPherson *et al.* 2006). Motif 5 was an additional variation of the CGG binding element containing CGG and GCG triplets. Lastly, motif 6 (CCTCGG) differed by one nucleotide from the putative nitric oxide-responsive element (NORE) motif of Cta4 (CCGTCGG), which also belongs to the same zinc cluster transcription regulatory family as Zcf2 (CHIRANAND *et al.* 2008). The motifs are listed in Table 4.

	Reason	Location in *SSU1* promoter	Sequence and mutation
Motif 1	MEME	-357	**G**A**CA**AA**A**AGAGGAGAGAGA**G**A**GGC** T AG C T TTA
Motif 2	MEME	-843	**A**CG**T**G**A**CT**T**GA**C** G C G G T
Motif 3	MEME	-662	**T**GC**TTG** G CCA
Motif 4	Gal4	-395	**CGG**TAATAAACGTATAC**CCG** ATT AAT
Motif 5	Gal4	-240	**CGG**TGTGTCACAAT**C**T**GCG** ATT A TAA
Motif 6	NORE	-259	TTT**T**T**C**C**T**CGG G A G
Motif 4A	Motif 4 phenotype	-412	**CGG** ATT
Motif 4B	Motif 4 phenotype	-395	**CCG** AAT

Table 4- Mutated motifs located within 1-kb upstream of *SSU1.*

No effect was observed when motifs 1-3 and 5 were mutated, however, mutating motifs 4 and 6 reduced *SSU1-gLUC* induction, respectively (Figure 18). Additionally, mutating the motif 4 triplets (CGG and CCG) individually (motif 4A and motif4B) also resulted in reduced *SSU1-gLUC* induction by nitrite (Figure 18B). Thus, two putative zinc cluster regulatory sites have been identified that regulate *SSU1* in response to sulfite and nitrite.

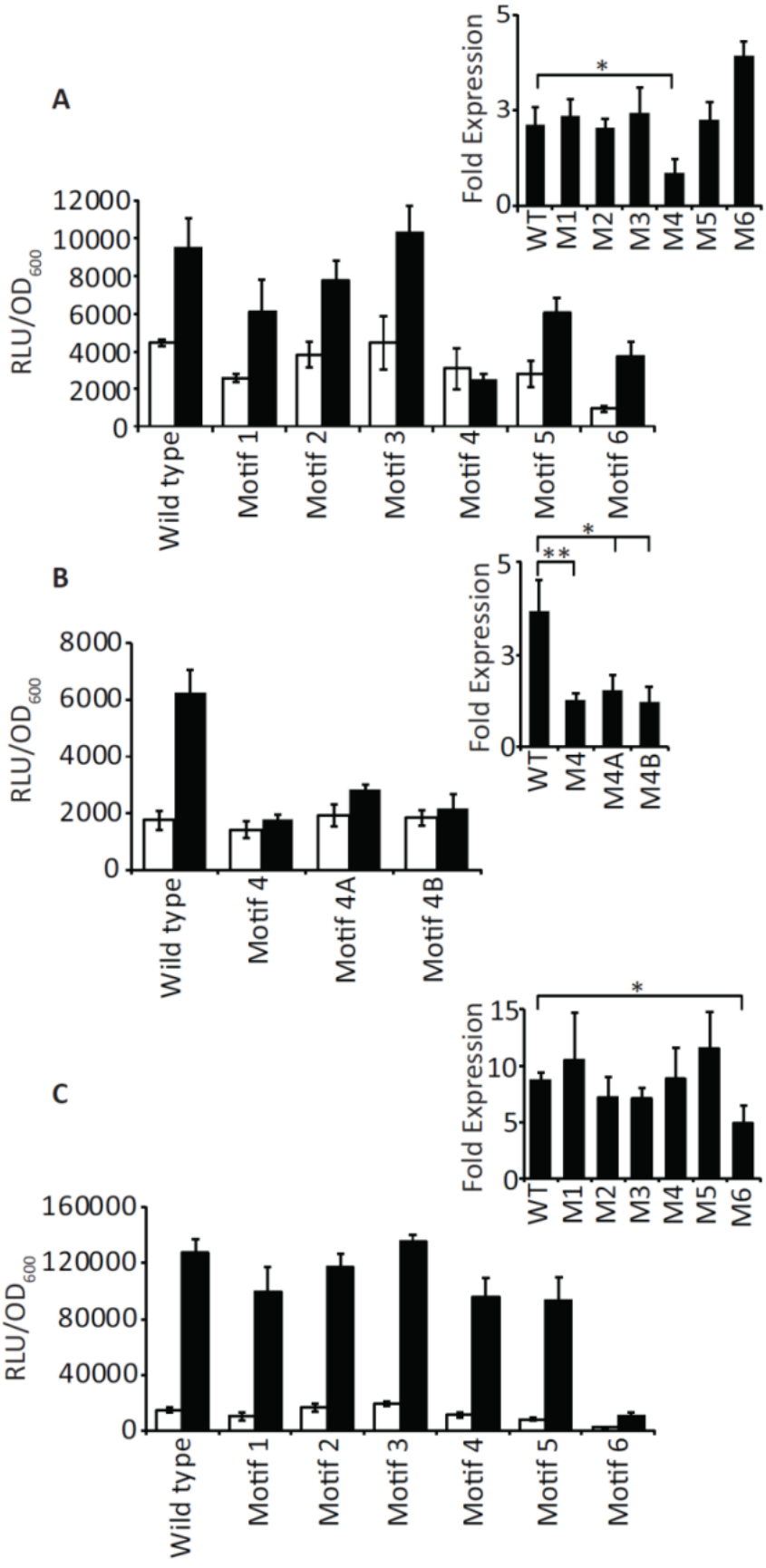

Figure 18- Mutating motifs 4 and 6 reduced *SSU1-gLUC* induction by nitrite and sulfite, respectively. Cultures in log phase were grown in the presence (black) or absence (white) of **(A & B)** 0.5 mM nitrite for 45 minutes, or **(C)** 1.0 mM sulfite for 30 minutes, at 30°C. The insets shown in **(A)(B),** and **(C)** show fold expression change using the ratio: ((RLU/OD600inducer) /(RLU/OD600control)). Results are averages of three biological replicates. (*) and (**) asterisks indicate that the detected differences were significant: (P<0.05) and (P<0.01), respectively.

9.2 Investigating localization of Zcf2 and Cta4 in response to sulfite using microscopy.

Protein function can be elucidated through the study of protein subcellular localization and complexing interactions. Localization and protein complex formations can be determined using fluorescent microscopy based techniques. To further examine the functions of the zinc-cluster transcription factors Zcf2 and Cta4, green fluorescent protein (GFP) was fused to both the N-terminus and C-terminus of Zcf2, and the C-terminus of Cta4. This would allow for the examination of regulation and cross-regulation of these TFs by sulfite or nitrite. The hypothesis was that the TFs will reside in the cytoplasm of unstressed cells, and then move to the nucleus after induction by a RNS or RSS. A well-studied example of this mechanism is seen with the heat shock transcription factor HSF1, where HSF1 is distributed in the cytoplasm in unstressed cells, and then redistributed to specific areas of the nucleus after heat-stress (MORIMOTO 1998).

The *ZCF2-GFP*$_{(N\text{-}terminus)}$ construct was introduced in a *Δzcf2* mutant strain and resulted in complementation of the *Δzcf2* phenotype. The *ZCF2-GFP*$_{(C\text{-}terminus)}$ and *CTA4-GFP*$_{(C\text{-}terminus)}$ constructs were transformed in *Δzcf2/ZCF2* and *Δcta4/CTA4* heterozygotes, respectively. When *C. albicans* strains containing the chimeric proteins Zcf2-GFP and Cta4-GFP were exposed to sulfite or nitrite, respectively, and visualized under a fluorescence microscope, no fluorescence was observed. It is possible no fluorescence was observed because the genes for the fluorescent proteins used to tag the transcription factors were placed out of the correct reading frame of the genes encoding the TFs. Another possibility is that the TFs are generally expressed in low quantities in cells and are therefore very hard to visualize using GFP as the fluorescent proteins. I briefly discuss future experiments that could be done to get around these issues in Chapter 12 of this thesis.

9.3 Investigating Zcf2 and Cta4 binding motifs using a Chip-exo analysis.

Transcription factor DNA binding sites are short DNA sequences, typically 4 to 30 base pairs long, that usually differ in affinity for different transcription factors. DNA binding sites also have the potential to undergo fast evolutionary change (BORNEMAN *et al.* 2007). Zcf2 and Cta4 are known to activate several genes involved in sulfite and nitric oxide stress, as well as nitrogen catabolism. To determine the binding sites of these two TFs, ChIP-exo, a chromatin immunoprecipitation method, was used. ChIP-exo achieves near single-base resolution, and involves the use of an exonuclease that trims ChIP DNA to a precise distance from the crosslinking sites, followed by deep sequencing of bound locations detectable as peak pairs (RHEE AND PUGH 2011). The ChIP-exo method has not yet been used for *C. albicans.* Cell pellets of *C. albicans* strains containing either 9x*MYC* or 3x*FLAG* tagged Cta4 and Zcf2 proteins, respectively, were sent to a ChIP-exo processing company for analysis. The strains containing the tagged Cta4 and Zcf2 proteins were exposed to sub-toxic levels of nitric oxide and sulfite, respectively. Results are currently being analyzed by an undergraduate researcher in our laboratory. Thus far, it appears that Zcf2 and Cta4 bind to multiple locations, and bind more locations when induced by sulfite and nitric oxide, respectively, when compared to control samples.

Chapter 10: Internal production of sulfite mediates resistance to external sulfite

Sulfite and sulfide are produced endogenously in *C. albicans* as metabolic intermediates in the sulfite assimilation pathway. In efficient pathways, products rapidly become substrates for the subsequent steps, while in other pathways, an overflow of

diffusible, toxic pathway intermediates can occur (HUANG *et al.* 2001). Several mechanisms limit the damage of these toxins and include compartmentation of the pathway, enzymatic conversion to nontoxic byproducts, or export of the toxic intermediate out of the cell (XU *et al.* 1994; KERFELD *et al.* 2010). Removal of toxic pathway intermediates by catalysis or transport is inducible by the respective intermediate or as part of a metabolic program for maintaining homeostatic levels of pathway enzymes. Accordingly, this suggests that *SSU1* expression is inducible by an overabundance of sulfite or sulfide produced by the sulfate assimilation pathways. I began to test this by growing *C. albicans* cells to log phase in rich medium, and then resuspending and growing the cells for an hour in minimal defined medium lacking amino acids.

Under these conditions, the expression of *MET3,* encoding the first enzyme of the sulfate assimilation pathway, was analyzed using a *MET3-gLUC* reporter where luciferase was placed under the regulation of the *MET3* promoter. Expression of *MET3* was induced by the minimal medium, and this induction was inhibited by adding methionine to the medium (Figure 19). This finding is consistent with an expected induction of the sulfate assimilation pathway in minimal medium, due to cellular requirements for the amino acids methionine and cysteine (methionine is also converted by cells into cysteine). After 1 hour of growth in this minimal medium, sulfite resistance was tested by switching *C. albicans* back to rich medium with or without added sulfite.

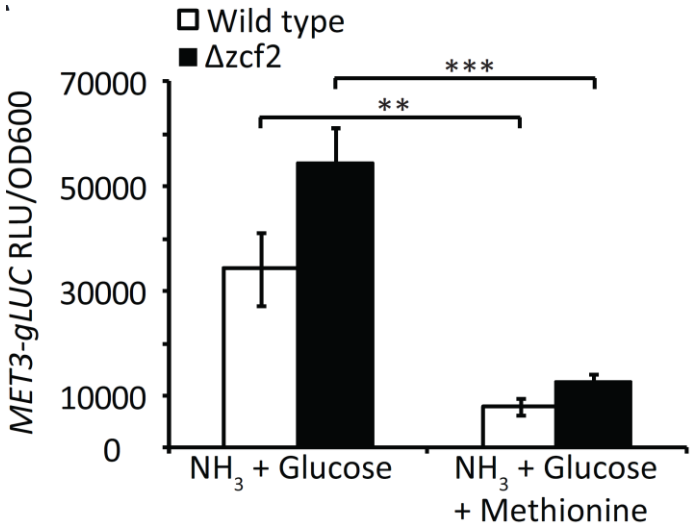

Figure 19- Increased sulfate assimilation pathway flux increases *MET3-gLUC* induction. *MET3-gLUC* reporter luminescence was measured in *C. albicans* wild type (white) and Δ*zcf2* (black) strains that were grown for one hour at 30°C in SD medium containing ammonium chloride and glucose, and containing or lacking methionine. Results are averages of three biological replicates. (**) and (***) asterisks indicate that the detected differences were significant: (P<0.01) and (P<0.001), respectively.

Addition of methionine to the short term minimal media culture, which reduces induction of the sulfate assimilation pathway, caused increased growth sensitivity to sulfite in rich medium. Accordingly, wild type cells grown without methionine were less sensitive to sulfite than the cells grown with methionine. This decrease in sensitivity to sulfite was not seen in a *Δmet16* mutant incapable of generating endogenous sulfite, indicating a role for endogenous sulfite production in sulfite resistance (Figure 20A).

Expression of *SSU1-gLUC* showed a similar pattern in minimal medium as *MET3-gLUC*, that is, a growth-dependent, methionine-inhibited, induction of expression that was significantly reduced in *Δmet16* or *Δzcf2* mutant strains (Figure 20B). Together these data predict a model in which excess endogenous sulfite produced by the Met16 PAPS reductase

is reduced by a Zcf2-dependent sulfite (and sulfide)-induction of the sulfite exporter Ssu1, with a corresponding decrease in sensitivity to exogenously added sulfite. Therefore, these results are consistent with a model in which endogenous sulfite produced via sulfate assimilation pathway flux promotes greater resistance to extracellular sulfite.

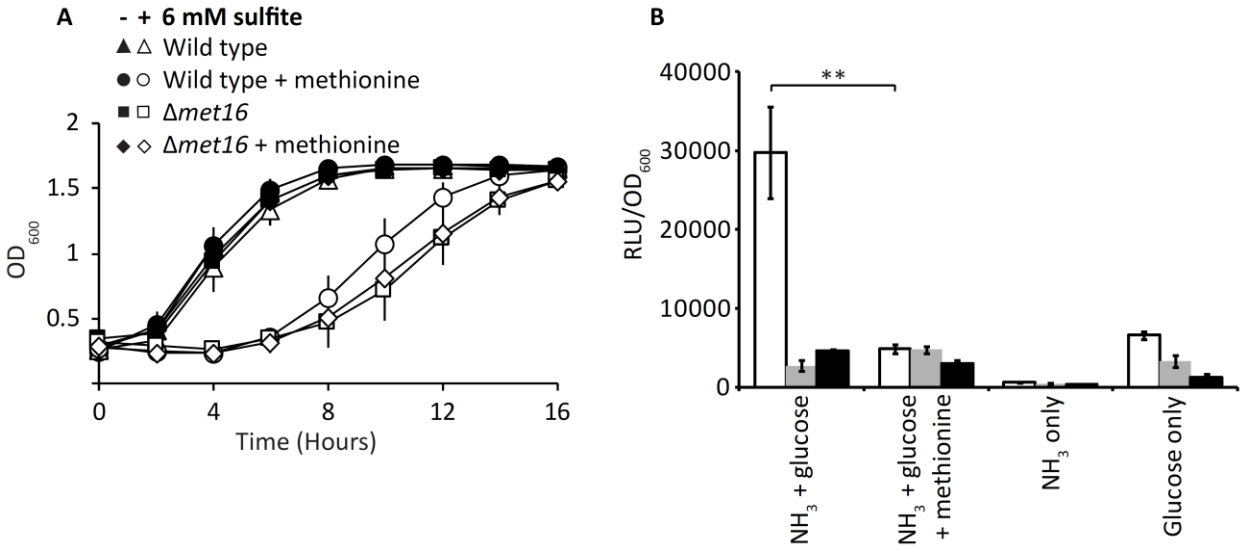

Figure 20-A) Increased sulfate assimilation pathway flux induces *C. albicans* sulfite resistance. Wild type and Δ*met16* mutant strains were challenged for 16 hours in YEPD pH4 with 0 mM and 6 mM sulfite (filled and empty markers, respectively) after being grown to log phase for 3 hours in YEPD pH4, and then in SD medium (supplemented with ammonium chloride and glucose) with or without methionine for 1 hour. Results are averages of 4 experiments, each with 3 biological replicates for each condition. **B) Increased sulfate assimilation pathway flux increases endogenous sulfite levels.** *SSU1-gLUC* reporter luminescence was measured in *C. albicans* wild type (white), Δ*zcf2* (gray), and Δ*met16* (black) strains that were grown for one hour at 30°C in SD medium containing or lacking ammonium chloride, glucose, and methionine. Results are averages of three biological replicates. (**) asterisks indicate that the detected differences were significant: (P<0.01). Error bars represent SD.

Chapter 11: Investigation of *Candida albicans* inhibition by *Veillonella parvula*

Throughout this document, I discuss *Candida albicans* abilities and mechanisms used to resist stress caused by RSS. It is useful to remind the reader that the context in which these findings may be relevant concern *C. albicans* interactions with diverse host-niches and neighboring bacteria. As such, I contributed to a colleague's manuscript by writing part of the discussion section regarding the relevance of his work regarding *C. albicans* interactions with oral anaerobic bacteria studied under anaerobic conditions:

One of the more challenging niches that this yeast must inhabit is the oral cavity of its human host which is an environment that contains areas of both high and low oxygen levels and is also colonized by a diverse bacterial microflora. These bacteria influence *C. albicans* abundance by affecting its adhesion to surfaces, hyphae growth, cell viability, and biofilm formation; mostly through diffusible chemicals (JAROSZ *et al.* 2009; JAMES *et al.* 2016). Research in our lab however has demonstrated that the oral anaerobe *Veillonella parvula* inhibits *C. albicans* hyphal and yeast growth through a contact-dependent mechanism. This interaction varies from several of those previously studied where bacteria adherence or co-aggregation with *C. albicans* enhanced colonization (CANNON AND CHAFFIN 1999; BAMFORD *et al.* 2009). This study also differs from previous ones showing bacteria contact-dependent inhibition of *C. albicans* because the experiments were done under anaerobic conditions. Moreover, it was suggested that metabolic exchanges also play a role in *C. albicans* interaction with *V. parvula.*

The inhibition of *C. albicans* by *V. parvula* occurred in the presence or absence of the *Streptococcus* spp., which are the preferred metabolic partners of *Veillonella; i.e., Veillonella*

spp. utilize Streptococcus-derived lactate for growth. However, *V. parvula* does not grow on ethanol, an end-product of sugar metabolism in *C. albicans.* Thus, these findings suggest a model in which *Veillonella* selects against *C. albicans* to favor competition for sugar resources by its metabolic partner *Streptococcus.*

Chapter 12: Conclusions and Future Directions

For decades, Reactive Oxygen Species and Reactive Nitrogen Species have dominated the field of oxidative stress research. However, in 2001, a review by Giles *et al.* suggested the recognition of an additional group of redox active molecules termed 'Reactive Sulfur Species' (GILES *et al.* 2001). This review examined decades of research regarding thiol, disulfide, and sulfur-radical chemistry in mammals, microbes, and plants, and defined RSS as "the potential of sulfur in higher oxidation states to be formed under conditions of oxidative stress, and for these RSS to act as further aggressive oxidizing agents". During that time, RSS were mostly known as transmitters of signals and as redox active molecules formed because of oxidative stress. However, since then it became apparent that RSS are also stressors themselves as they generally reduce thiols and thus directly or indirectly lead to an increase in ROS concentrations. The selective oxidation of thiols by RSS has been exploited in therapies against microbes, and is also being considered in the context of cancer therapy (GILES AND JACOB 2002; GRUHLKE *et al.* 2016). In this thesis, I discuss the response of the human pathogen *Candida albicans* to reactive sulfur species.

First I showed that *C. albicans* alters its sulfite sensitivity in response to different RSS found in its human host, as well as in response to nitrite pretreatment, showing

evidence for cross-protection. Zcf2 is necessary for *C. albicans* to respond to sulfite and sulfide which suggests that these RSS are ligands that are directly or indirectly sensed by Zcf2; this is a novel finding as a specific receptor for sulfide in yeast is previously not known (MISHANINA *et al.* 2015). Bacteria use the *bigR* operon for hydrogen sulfide detoxification; this involves the oxidation of hydrogen sulfide to sulfite by the sulfur dioxygenase Blh, followed by export of the toxic sulfite. The *bigR* operon is regulated by the winged-helix protein BigR, which represses transcription of its own operon. BigR derepresses transcription upon oxidation, however, the signals that modulate its activity are poorly understood and hydrogen sulfide is unlikely to be the oxidizing agent due to its redox potential (GUIMARAES *et al.* 2011). Instead, the authors proposed that hydrogen sulfide-induced reactive oxygen species could play a role in BigR oxidation. To examine whether Zcf2 is sensing sulfide or sulfide-induced reactive oxygen species, future experiments are needed examining Zcf2-conformational changes in response to sulfide and ROS. Moreover, even though *C. albicans* changes its sensitivity to sulfite in response to sulfite or sulfide, I was unable to observe adaptation to sulfide following the same pretreatment. This may be because the nonpolar hydrogen sulfide molecule, which is abundant at neutral pH with its pKA of 6.9, freely permeates the phospholipid bilayer. Therefore, unlike sulfite resistance which is correlated with an increase in expression of *SSU1,* encoding a sulfite transport protein, adjusting sulfide resistance by changing the activity of a transporter protein is unlikely to be effective.

Apart from sensing sulfite and sulfide, Zcf2 is required for cysteine inducible expression of *SSU1* (HENNICKE et al. 2013). However, when I used the *SSU1-gLUC* reporter to examine *SSU1* expression in response to cysteine, I showed that *SSU1* regulation by

cysteine is only partially Zcf2-dependent. *SSU1-gLUC* fold induction in response to cysteine was reduced in the *Δzcf2* strain compared to wild type, however, a significant increase in *SSU1* expression was still observed. This increase did not occur when sulfite or sulfide was added to the *Δzcf2* strain. Furthermore, when cysteine was added to a strain lacking *CDG1,* encoding the enzyme that converts cysteine to sulfite, *SSU1-gLUC* fold induction was reduced, suggesting that cysteine activation of *SSU1* is due to cysteine conversion to sulfite. Therefore, it is possible that the reduced levels of *SSU1* induction in the *Δzcf2* strain was because there was no Zcf2 present to sense the resulting sulfite formed from the added cysteine. The cysteine-induced increase in *SSU1-gLUC* induction in the *Δcdg1* strain, however, indicates that cysteine itself is also a signal. To examine the induction of other genes in response to reactive sulfur species, I elucidated the sulfite regulon using a microarray-based analysis.

About 1000 genes were shown to be regulated by sulfite, which is roughly one sixth of the protein coding genes reported in *C. albicans.* This large number of responsive genes indicates the possibility of a temporary inhibition of growth by the sulfite added, or that sulfur metabolism is involved in diverse cellular processes; this was demonstrated previously where genes involved in sulfur amino acid synthesis were upregulated during biofilm formation (GARCIA-SANCHEZ *et al.* 2004). The genes that were most strongly regulated by sulfite belonged to four functional groups: sulfite removal, alternative respiration, ammonia metabolism, and ribosome biogenesis. The genes involved in ribosome biogenesis were down-regulated after sulfite exposure, similarly to what occurs when yeast are under ROS stress and downregulate ribosome biogenesis most likely to direct energy resources for other cellular processes (LOAR *et al.* 2004). Unlike other zinc

cluster family members, such as Zcf29, which is involved in downregulating ribosome biogenesis genes following exposure to hydrogen peroxide, Zcf2 does not play a role in downregulating ribosome biogenesis genes in response to sulfite (ISSI *et al.* 2017). However, most of the sulfite removal and ammonia metabolism genes are Zcf2-dependent. When I examined the sulfite sensitivity of three of the most upregulated genes in wild type response to sulfite, *AOX2, AMO1,* and orf19.3120, no phenotype was observed. *SSU1* was the only upregulated gene that, when deleted, results in a sulfite sensitive phenotype. Because orf19.3120 has yet to be characterized and has been shown to be highly upregulated in response to both sulfite and nitric oxide, I suggest that future studies need to be done to determine its function.

The most induced gene in response to sulfite in both wild type and the *Δzcf2* strain was *AOX2,* encoding a mitochondrial enzyme that provides an alternative oxidase pathway from cytochrome c oxidase. This suggests that sulfite is inhibiting respiration or cytochrome c oxidase. However, a well-known inhibitor of cytochrome c oxidase is sulfide, which is formed from sulfite by sulfite reductase, Ecm17, in the sulfate assimilation pathway (KHAN *et al.* 1990; NICHOLLS *et al.* 2013). Therefore, it is possible that the conversion of sulfite to sulfide results in the induction of *AOX2,* which is consistent with the finding that enzymatic conversion of sulfite to sulfide contributes to sulfite-induced toxicity.

The *C. albicans ∆ecm17* mutant is deficient in forming sulfite from sulfide and is less sensitive to sulfite; supporting the hypothesis that sulfite-derived sulfide is one mechanism of sulfite toxicity. To examine if this was because of increased *SSU1* levels in the *∆ecm17* mutant due to endogenous sulfite accumulation, I tested the sulfite sensitivity of a double

mutant *Δecm17 Δmet16*, which has lower levels of *SSU1* than *Δecm17.* The *Δecm17 Δmet16* strain showed a sulfite sensitivity phenotype that was intermediate between the wild type and *Δecm17* mutant, suggesting that both sulfite and sulfide are directly toxic to *C. albicans.* When I examined the effect of deleting the *ECM17* homolog, *MET5,* from *S. cerevisiae,* I did not observe a sulfite resistant phenotype. This observation is consistent with the difference in genes most upregulated in response to sulfite (*PDC1* in *S. cerevisiae* and *AOX2* in *C. albicans*), and suggests that these two yeasts tolerate sulfite toxicity in dissimilar ways. Another difference is that carbohydrate metabolism genes were induced in *S. cerevisiae* in response to sulfite, while nitrogen metabolism genes were induced in *C. albicans;* the induction of this gene set is not easily understood.

Despite using nitrogen-rich YEPD media when analyzing the transcriptional response of *C. albicans* to sulfite, several genes were upregulated that are generally involved in the response to nitrogen-starvation. One hypothesis is that *S*-sulfocysteine might be formed from the reaction of free cysteines with the exogenously added sulfite. *S*-sulfocysteine structurally resembles glutamate and is an antagonist of glutamate dehydrogenases, which are enzymes shown in yeast to regulate glutamate biosynthesis and balanced utilization of α-ketoglutarate, an important metabolite in aerobic respiration (OLNEY *et al.* 1975; DELUNA *et al.* 2001; ZHANG *et al.* 2004). To investigate this, future studies are required examining the induction of nitrogen catabolism genes in response to *S*-sulfocysteine. Examining the induction of metabolic pathways is also relevant for determining their roles in resistance to toxic metabolites.

The regulation of levels of toxic intermediates produced in metabolic pathways is poorly studied and in *C. albicans* there are no known enzymes that convert sulfite to non-

toxic sulfate, such as sulfite oxidase, which is found in humans, bacteria, and plants (KAPPLER AND ENEMARK 2015). Thus, *C. albicans* controls the levels of excess toxic sulfite in a different way that mainly involves getting rid of it via Ssu1.This was demonstrated by the finding that increasing sulfate assimilation pathway flux resulted in an increased resistance to exogenously added sulfite, and both increasing SAP flux and adding sulfite to *C. albicans* were correlated with an increase in *SSU1* induction. These results also suggest that similar mechanisms are used to avoid endogenous and exogenous sulfite toxicity.

Future experiments that will identify the subcellular locations of key proteins involved in sulfite resistance, such as Ssu1 and Zcf2, will also be useful in elucidating key aspects of their functions. Moreover, being able to visualize the location of Zcf2 within cells will provide more insight on how it regulates sulfide and sulfite. For example, it is not known whether these RSS regulate Zcf2 activity in the nucleus, or if Zcf2 senses these molecules in the cytoplasm before translocating to the nucleus to regulate gene activity. Previously *TAC1,* which is also a member of the zinc cluster family of transcription factors, was successfully labelled at the C-terminus with GFP (COSTE *et al.* 2004). The Tac1p-GFP fusion was under the control of the constitutively active *ACT1* promoter. Perhaps a similar technique could be used to visualize the location of the Zcf2 protein, however, one big drawback is that specific induction of the protein cannot be studied due to its regulation under the *ACT1* promoter. Alternatively, both Ssu1 and Zcf2 can be tagged with red fluorescent proteins such as DsRFP or mCherry, which should provide an improved contrast to GFP which is hampered by the green autofluorescence of *C. albicans* (GERAMI-NEJAD *et al.* 2009). Moreover, fluorescent proteins brighter than GFP can also be used such

as NeonGreen which is the brightest monomeric green or yellow fluorescent protein yet described, and is 3 times brighter than GFP (SHANER *et al.* 2013).

Closing Remarks

Candida albicans is an important fungal pathogen that is becoming increasingly resistant to existing antifungal therapy. I have described how this yeast can tolerate stress caused by Reactive Sulfur Species, a group of molecules that has been gaining increasing interest from many cellular biologists over the past two decades. I show that a member of the Zinc Cluster transcription factor family is necessary and sufficient for *C. albicans* resistance to sulfite. Members of this family experience higher rates of evolution and are predicted to play key roles in recent adaptation. I have also shown that *C. albicans* has a unique response to sulfite that is separate from the general oxidative stress response, and that the same mechanism mediates resistance to both endogenously produced and exogenously encountered sulfite.

Moreover, this work has elucidated potential protein drug targets that can be used to combat future *Candida* infections. For example, inhibiting Met16, the enzyme in the sulfate assimilation pathway that produces sulfite, causes *C. albicans* to be more sensitive to the toxic intermediates in that pathway. I have also demonstrated that growth conditions influence *C. albicans* resistance to sulfite, suggesting that diet supplementation can affect *Candida* growth. For example, growing *C. albicans* in the presence of extra methionine causes it to be less resistant to sulfite. Therefore, using this model, supplementing diets with amino acids that inhibit certain *C. albicans* biochemical pathways may cause *C. albicans* to be more sensitive to the toxic metabolite intermediates that it

produces endogenously, and thus might cause it to be a weaker pathogen. Further research is needed to confirm this.

Overall, these findings demonstrate a mechanism of *C. albicans* for adapting to stress using cues consisting of sub-toxic doses of 'poisons' to defend against an assault by larger doses.

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Appendix A: Strains used in this study

Strain	Parental Strain	Genotype	Source
Wild type	SN152	*Δarg4/Δarg4* *LEU2/Δleu2* *HIS1/Δhis1* *URA3/Δura3::imm434* *IRO1/Δiro1::imm434*	(HOMANN *et al.* 2009)
Δzcf2/Δzcf2	SN152	*Δzcf2* ::*CmLEU2/ Δzcf2*::*CdHIS1* *Δarg4/Δarg4* *URA3/Δura3::imm434* *IRO1/Δiro1::imm434*	(HOMANN *et al.* 2009)
ZCF2-3xFLAG	*Δzcf2/Δzcf2*	*RPS1/RPS1::CIp10-ZCF2p-3xFLAG-ZCF2*	This study
ZCF2-3xFLAG-hyperactive	*Δzcf2/Δzcf2*	*RPS1/RPS1::CIp10-ZCF2p-3xFLAG-hyperactive-ZCF2*	This study
ZCF2-GFP$_{(N\text{-terminus})}$	*Δzcf2/Δzcf2*	*RPS1/RPS1::CIp10-ZCF2p-GFP-ZCF2*	This study
ZCF2-GFP$_{(C\text{-terminus})}$	*Δzcf2/ZCF2*	*Δzcf2* ::*CmLEU2/ ZCF2-GFP* *Δarg4/Δarg4* *URA3/Δura3::imm434* *IRO1/Δiro1::imm434*	This study
CTA4-GFP$_{(C\text{-terminus})}$	*Δcta4/CTA4*	*Δcta4*::*CmLEU2/ CTA4-GFP* *Δarg4::dpl200/ Δarg4::dpl200*	This study

		Δleu2::dpl200/ Δleu2::dpl200 *Δhis1::dpl200/ Δhis1::dpl200* *URA3/ Δura3*::*imm*^{434} *IRO1/ Δiro1*::*imm*^{434}	
CTA4-9xMYC	*Δcta4/CTA4*	*Δcta4*::*CmLEU2/ CTA4-9xMYC-*(*ACT1*-3'UTR)-*CdHIS1* *Δarg4::dpl200/ Δarg4::dpl200* *Δleu2::dpl200/ Δleu2::dpl200* *Δhis1::dpl200/ Δhis1::dpl200* *URA3/ Δura3*::*imm*^{434} *IRO1/ Δiro1*::*imm*^{434}	(CHIRANAND *et al.* 2008)
Wild type- Arg^{+}	Wild type	*Δarg4/Δarg4*::*CaARG4* *LEU2/Δleu2* *HIS1/Δhis1* *URA3/Δura3::imm434* *IRO1/Δiro1::imm434*	This study
Δzcf2/Δzcf2- Arg^{+}	*Δzcf2/Δzcf2*	*Δzcf2* ::*CmLEU2/ Δzcf2*::*CdHIS1* *Δarg4/Δarg4::CaARG4* *URA3/Δura3::imm434* *IRO1/Δiro1::imm434*	This study
Δssu1/Δssu1	SC5314	*Δssu1::FRT/Δssu1::FRT*	(CHIRANAND *et al.* 2008)

Δssu1/Δssu1::SSU1	*Δssu1/Δssu1*	*Δssu1::FRT/Δssu1::SSU1-Nour^{R}*	(CHIRANAND *et al.* 2008)
Δecm17/Δecm17	SN152	*Δecm17* ::*CmLEU2/ Δ ecm17*::*CdHIS1* *Δarg4/Δarg4* *URA3/Δura3::imm434* *IRO1/Δiro1::imm434*	(NOBLE *et al.* 2010)
Δmet16/Δmet16	Wild Type	*Δmet16* ::*CdARG4/ Δecm17*::*CaHYGB* *URA3/Δura3::imm434* *IRO1/Δiro1::imm434*	This study
Δecm17/Δecm17 *Δmet16/Δmet16*	*Δecm17/Δecm17*	*Δmet16* ::*CdARG4/ Δecm17*::*CaHYGB* *URA3/Δura3::imm434* *IRO1/Δiro1::imm434*	This study
Δcta4/Δcta4	SN152	*Δcta4*::*CmLEU2/ Δcta4*::*CdARG4* *Δarg4::dpl200/ Δarg4::dpl200* *Δleu2::dpl200/ Δleu2::dpl200* *Δhis1::dpl200/ Δhis1::dpl200* *URA3/ Δura3*::*imm^{434}* *IRO1/ Δiro1*::*imm^{434}*	(CHIRANAND *et al.* 2008)
Δcdg1/Δcdg1	SN152	*Δcdg1*::*CmLEU2/ Δcdg1*::*CdHIS1* *Δarg4/Δarg4* *URA3/Δura3::imm434* *IRO1/Δiro1::imm434*	(NOBLE *et al.* 2010)

Wild type-*SSU1-gLUC*	Wild Type	*RPS1/RPS1::CIp10-SSU1p-PGA59-gLUC*	This study
Wild type-*ACT1-gLUC*	Wild Type	*RPS1/RPS1::CIp10-ACT1p-PGA59-gLUC*	This study
Δzcf2/Δzcf2-SSU1-gLUC	*Δzcf2/Δzcf2*	*RPS1/RPS1::CIp10-SSU1p-PGA59-gLUC*	This study
Δzcf2/Δzcf2-ACT1-gLUC	*Δzcf2/Δzcf2*	*RPS1/RPS1::CIp10-ACT1p-PGA59-gLUC*	This study
Δecm17/Δecm17-SSU1-gLUC	*Δecm17/Δecm17*	*RPS1/RPS1::CIp10-SSU1p-PGA59-gLUC*	This study
Δecm17/Δecm17-ACT1-gLUC	*Δecm17/Δecm17*	*RPS1/RPS1::CIp10-ACT1p-PGA59-gLUC*	This study
Δmet16/Δmet16-SSU1-gLUC	*Δmet16/Δmet16*	*RPS1/RPS1::CIp10-SSU1p-PGA59-gLUC*	This study
Δmet16/Δmet16-ACT1-gLUC	*Δmet16/Δmet16*	*RPS1/RPS1::CIp10-ACT1p-PGA59-gLUC*	This study
Δecm17/Δecm17 Δmet16/Δmet16-SSU1-gLUC	*Δecm17/Δecm17 Δmet16/Δmet16*	*RPS1/RPS1::CIp10-SSU1p-PGA59-gLUC*	This study
Δecm17/Δecm17 Δmet16/Δmet16-ACT1-gLUC	*Δecm17/Δecm17 Δmet16/Δmet16*	*RPS1/RPS1::CIp10-ACT1p-PGA59-gLUC*	This study

Δcta4/Δcta4-SSU1-gLUC	*Δcta4/Δcta4*	*RPS1/RPS1::CIp10-SSU1p-PGA59-gLUC*	This study
Δcta4/Δcta4-ACT1-gLUC	*Δcta4/Δcta4*	*RPS1/RPS1::CIp10-ACT1p-PGA59-gLUC*	This study
Δcdg1/Δcdg1-SSU1-gLUC	*Δcdg1/Δcdg1*	*RPS1/RPS1::CIp10-SSU1p-PGA59-gLUC*	This study
Δcdg1/Δcdg1-ACT1-gLUC	*Δcdg1/Δcdg1*	*RPS1/RPS1::CIp10-ACT1p-PGA59-gLUC*	This study

Appendix B: Primers used in this study

Name	**Sequence***
Primers to delete orf19.3120	
Primer 1	CAAGATAGTTTTTCGTAGATCGTATAC
Primer 3	CACGGCGCGCCTAGCAGCGGGCAACAGGGCCCATTTATAATC
Primer 4	GTCAGCGGCCGCATCCCTGCCTCGTACAGAAATGTACTCTTTACG
Primer 6	TACTCCTTGTCCATACTGGGATAC
LEU2 and *HIS1* Universal primer 2	CCGCTGCTAGGCGCGCCGTGACCAGTGTGATGGATATCTGC
LEU2 and *HIS1* Universal primer 5	GCAGGGATGCGGCCGCTGACAGCTCGGATCCACTAGTAACG
HygromycinB 2 Forward	CCGCTGCTAGGCGCGCCGTGCTCTCGGTACCAGAGCTATTAAGATCACCA GCCT
HygromycinB 5 Reverse	GCAGGGATGCGGCCGCTGACCTCTTTCTAGAATTTTATGATGGAATGAA TGGGATG
ARG4 2 Forward	CCGCTGCTAGGCGCGCCGTGCTGGTACTAAAGTATACACAC
ARG4 5 Reverse	GCAGGGATGCGGCCGCTGACCACTTTTAGCAGTACCACCA
Primers to delete *AMO1*	
Primers for *LEU2* and *HIS* are the same as the primers used to delete orf19.3120	
Primer 1	TGGGGCTTAAATACAATTGTGACC
Primer 3	CACGGCGCGCCTAGCAGCGGTGATGTTGAAGAAAACTTGGTGAGG
Primer 4	GTCAGCGGCCGCATCCCTGCTGTGTTGTTTGATATGTAATGAGAGAGT
Primer 6	CAGCTGGATCCAACAAACACACTT
Primers to delete *MET16*	
Primers for HygromycinB and *ARG4* are the same as the primers used to delete *SSU1*	
Primer 1	TGTGGTAACACCTTACTGGGAATC
Primer 3	CACGGCGCGCCTAGCAGCGGTGCTTATGTGTGTAGTGCTTGCT
Primer 4	GTCAGCGGCCGCATCCCTGCTCGATCCAGTCCTTGATAAGCA
Primer 6	CAACTTCTTATACTTGCGATGACG
Primers for constructing *ZCF2-GFP* (N-terminus)	
ZCF2 promoter Forward	GCACTATTTGATTCTAATGCAAATTAAC
ZCF2 promoter Reverse	TTTCTTAGTGAGGATTATAGATGAATAGA
ZCF2 ORF + 3'UTR Forward	ATGCTGGAAAAAATTAAGAGAGAAAAGCC
ZCF2 ORF + 3'UTR Reverse	TCTTCTGTAAGGTGCACGGGTTTTCTT
CIp10-*NAT* Forward	CCGTGCACCTTACAGAAGA CAG CTT TTG TTC CCT TTA GTG
CIp10-*NAT* Reverse	TTTGCATTAGAATCAAATAGTGC ACG CGT AGA TCC AAC TCA AGT
GFP Forward	CATCTATAATCCTCACTAAGAAA AAGCTTTATTAAAATGTCTAAAGGTG
GFP Reverse	TCTCTCTTAATTTTTTCCAGCAT TTATTTGTACAATTCATCCATACC

Primers for constructing *ZCF2-GFP* (C-terminus)	
1st fragment (end of *ZCF2* minus stop codon)	
1-Forward	CAA gggccc TTGACTGCAATTTATCCCATTTCC
2-Reverse	CAA gatatccatc TCTAAAGTGGAAGGAAAAGGATCA
2nd fragment (*ZCF2* + 3'UTR)	
3-For	CAA gagctc ATGTTTACAAGAACTAAATAATATTG
4-Rev	CAA ggtacc AACTTTTTCAAATTTACTCCATG
Primers for constructing *ZCF2-3xFLAG*	
Primers for *ZCF2* promoter, *ZCF2* ORF + 3'UTR, and CIp10-*NAT* are the same as the primers used to construct *ZCF2-GFP* (N-terminus)	
3xFLAG Forward	CATCTATAATCCTCACTAAGAAAATGGATTATAAAGATCACGATGG
3xFLAG Reverse	TCTCTCTTAATTTTTTCCAGCATAGCACCACCTTTATCATCATCT
Primers for *MET3-gLUC*	
MET3 promoter Forward	CAA ctcgag CATTCCCTTGGGAATTATACTGAGTAATGG
MET3 promoter Reverse	CAA aagctt GTTTTCTGGGGAGGGTATTTACTTTTA
Primers to mutate *SSU1* promoter	
DNA Fragment 1 Forward	ggtaccCTTGAAACCAAGAGGAAAATC
DNA Fragment 2 Reverse	CATccaaatcggtggTAGC
Motif 1 Fragment 1 Reverse	**TAA**T**A**TCTCTCTCCTCT**G**TT**CT**T**A**TAAGTTTGTTTTCACGGGTATA
Motif 1 Fragment 2 Forward	**T**A**AG**AA**C**AGAGGAGAGAGA**T**A**TTA**TTGATTACAGTTAATCTTCTACA
Motif 2 Fragment 1 Reverse	**A**TC**C**AG**CC**G**C**G**C**GAACTTTCATCATCCCGTAA
Motif 2 Fragment 2 Forward	**G**CG**C**G**G**CT**G**GA**T**TGGCATATTGTGATTATAAATTCT
Motif 3 Fragment 1 Reverse	A**TGG**GC**C**CAAACTGCATATAGTGTATGT
Motif 3 Fragment 2 Forward	G**G**GC**CCA**TGCGCACTACCTTTTTTTTC
Motif 4 Fragment 1 Reverse	**ATT**GTATACGTTTATTA**AAT**TGATTATTTCTTAAACCCATAAG
Motif 4 Fragment 1 Forward	**ATT**TAATAAACGTATAC**AAT**TGAAAACAAACTTAGACAAAAAG
Motif 5 Fragment 1 Reverse	**TTA**A**T**ATTGTGACACA**AAT**AGGAAAAATCTAGCCTAAATTAAG
Motif 5 Fragment 2 Forward	**ATT**TGTGTCACAAT**A**T**TAA**AGTGATTCAAAGTCCTCTTC
Motif 6 Fragment 1 Reverse	CCG**C**G**T**A**C**AAATCTAGCCTAAATTAAGTGC
Motif 6 Fragment 2 Forward	TTT**G**T**AC**G**C**GGTGTGTCACAATCTGC

*Restriction sites are shown in lower case letters. Underlined sequences denote overlapping oligo primers. Bold sequences indicate nucleotide changes or mutations introduced.